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FILING DATE: *January 14, 2003*
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By Authority of the
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01-16-03

PTO/SB/16 (10-01)

Approved for use through 10/31/2002. OMB 0651-0032

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV089312165US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☑ Additional inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Compositions and Methods for Detection and Isolation of Phosphophorylated Molecules

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification Number of Pages 152

☑ Drawing(s) Number of Sheets 14

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

☑ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 13-3900

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☑ Yes, the name of the U.S. Government agency and the Government contract number are: National Cancer Institute 1 R33 CA093292-01

Respectfully submitted,

SIGNATURE Koren J. Anderson

Date 1/14-03

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Docket Number:

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PTO/SB/17 (10-01)
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FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	
Filing Date	01/14/2003
First Named Inventor	Brian Agnew, et al.
Examiner Name	
Group Art Unit	
Attorney Docket No.	

METHOD OF PAYMENT

1. ☑ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

Deposit Account Number: 13-3900

Deposit Account Name: Molecular Probes, Inc.

☑ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

☑ Applicant claims small entity status. See 37 CFR 1.27

2. ☐ Payment Enclosed:

☐ Check ☐ Credit card ☐ Money Order ☐ Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES

	Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		x =
Independent Claims		- 3** =		x =
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
Other fee (specify)					

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 0.00

SUBMITTED BY

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Number 1 of 1

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Signature Judith M. Ubando

Printed Name Judith M. Ubando

COMPOSITIONS AND METHODS FOR DETECTION AND ISOLATION OF PHOSPHORYLATED MOLECULES

INTRODUCTION

Statement Regarding Federally Sponsored Research

This invention was made in part with government support under grant number 1 R33 CA093292-01, awarded by the National Cancer Institute. The United States Government may have certain rights in this invention.

Field of the Invention

The present invention relates to metal-chelating compositions and methods for use in the detection and isolation of phosphorylated target molecules. The invention has applications in the fields of proteomics, molecular biology and diagnostics.

Background of the Invention

Phosphorylation and dephosphorylation are processes by which phosphate groups are added or removed from a target molecule, typically a protein. The process of reversible phosphorylation is a key feature of cellular regulation, including signal transduction, gene expression, cell cycle regulation, cytoskeletal regulation and apoptosis. See, *e.g.*, PROTEIN PHOSPHORYLATION (Marks F. ed., 1996); Hunter, "Signaling – 2000 and beyond," *Cell 100*:113-127 (2000). Principally, two classes of enzymes (kinases and phosphatases) modulate reversible protein phosphorylation, adding phosphate groups and removing phosphate groups, respectively, from molecules. Phosphorylation reactions are key features of protein function, and thus phosphorylated proteins must be able to be identified if the proteome is to be fully understood; however, to date no practical methods exist for the systematic parallel analysis of the phosphorylation status of large sets of proteins involved in the regulatory circuitry of cells and tissues. See, Wilkins *et al., Genetic Eng. Rev. 13*:19 (1995).

Signal transduction is an example of a process involving protein phosphorylation that is critical for cellular regulation. After an extracellular stimulatory factor binds to its recognized cell surface receptor, signal transduction is initiated, often by a specific set of cellular protein kinases. These kinases subsequently phosphorylate the target molecule, resulting in an altered activity and a continued cellular response to the signal. *See, e.g.,* Nishizuka, "Studies

and perspectives of protein kinase C," *Science 233*:305-312 (1986). It is not enough for researchers to simply identify whether a protein is a phosphorylated protein or not. It has become additionally essential for researchers to identify the sites of phosphorylation on proteins. Serine, threonine and tyrosine amino acid residues are the most common sites of phosphorylation in eukaryotic cells. *See, e.g.*, Guy *et al.* "Analysis of Cellular Phosphoproteins by Two-Dimensional Gel Electrophoresis: Applications for Cell Signaling in Normal and Cancer Cells," *Electrophoresis 15*:417-440 (1994). Thus, the focus for researchers in understanding protein phosphorylation events occurs at two levels. The first level of analysis requires a determination of *whether* a protein is a phosphoprotein, including identifying molecules responsible for phosphorylation, and the second level of analysis requires the identification of *which* amino acid is phosphorylated. The present invention provides materials and methods for both levels of analysis. The present invention also provides materials and methods for analysis of certain other phosphate and thiophosphate-containing materials including esters of carbohydrates, nucleotides and lipids.

Currently, phosphoproteins are most often detected by autoradiography after incorporation of ^{32}P or ^{33}P into cultured cells or after incorporation into subcellular fractions by protein kinases. *See, e.g.*, Yan et al., "Protein Phosphorylation: Technologies for the Identification of Phosphoamino Acids," *J. Chromatogr. A. 808*:23-41 (1998); Guy, G., Phillip, R. and Tan, Y. Electrophoresis 15, 417-440 (1994). Such approaches are restricted to a limited range of biological materials, such as tissue culture samples and analysis of clinical samples would require *in vivo* labeling of patients, which is not feasible. Several alternatives to radiolabeling have also been developed over the years.

Phosphoproteins can also be detected by immunoblotting and immunoprecipitation. *See, e.g.*, Soskic *et al.*, "Functional Proteomics Analysis of Signal Transduction Pathways of the Platelet-Derived Growth Factor Beta Receptor," *Biochemistry 38*:1757-1764 (1999); Watty *et al.*, "The In Vitro and In Vivo Phosphotyrosine Map of Activated MuSK," *Proc. Natl. Acad. Sci. USA. 97*:4585-4590 (2000). Immunoblotting is best performed after blocking unoccupied sites on the solid-phase support with protein solutions, which interferes with microchemical analysis. Removal of the antibody and stain require relatively harsh treatments (i.e., heating to 65°C, incubation with 0.1% SDS and 1 mM DTT). This also poses problems with subsequent use of the protein for sequencing and mass spectrometry. For immunoprecipitation, only the anti-phosphotyrosine antibodies display binding that is tight enough to allow effective isolation. Though high-quality antibodies to phosphotyrosine are commercially available, antibodies that specifically recognize phosphoserine and phosphothreonine residues have been more problematic, often being sensitive to amino acid

sequence context. The reliability of these antibodies has been questioned because of potential steric hindrances between the interaction of these antibodies and the phosphoproteins. Moreover, when phosphoproteins are not enriched prior to detection with the antibody, the presence of unrelated proteins co-migrating with the protein of interest may lead to false positive signals. Therefore, identification of phosphorylated proteins using immunoblotting and immunoprecipitation techniques is limited to proteins containing phosphorylated tyrosine residues. *See* McLachlin & Chait, *supra*.

Alternatively, phosphorylated proteins can be identified using chromogenic dyes, but with limited success. The cationic carbocyanine dye "Stains-All" (1-ethyl-2-[3-(3-ethylnaphtho[1,2d]thiazolin-2-ylidene)-2-methylpropenyl]-naphtho[1,2d]thiazolium bromide) stains RNA, DNA, phosphoproteins and calcium-binding proteins blue while unphosphorylated proteins are stained red. *See, e.g.*, Green *et al.*, "Differential Staining of Phosphoproteins on Polyacrylamide Gels with a Cationic Carbocyanine Dye," *Anal. Biochem. 56*:43-51 (1973); Hegenauer *et al.*, "Staining Acidic Phosphoproteins (Phosvitin) in Electrophoretic Gels," *Anal. Biochem. 78*:308-311 (1977); Debruyne, "Staining of Alkali-Labile Phosphoproteins and Alkaline Phosphatases on Polyacrylamide Gels," *Anal. Biochem. 133*:110-115 (1983); "Staining of phosphoproteins in polyacrylamide gels with acridine orange", *Seikagaku* 45:327-35 (1973). Stains-All is not routinely used to detect phosphoproteins due to poor specificity and low sensitivity. Stains-All is at least 10 times less sensitive than Coomassie Brilliant Blue as a general protein stain and several orders of magnitude less sensitive than ^{32}P-autoradiography or the techniques described in this patent. Another chromogenic method, the GelCode™ Phosphoprotein detection kit (Pierce Chemical Company, Rockford, IL), is designed to detect phosphoproteins in gels; however, this method has many limitations. According to this method, phosphoproteins are detected in gels through alkaline hydrolysis of phosphate esters of serine or threonine, precipitation of the released inorganic phosphate with calcium ions, formation of an insoluble phosphomolybdate complex and then visualization of the complex with a dye such as methyl green, malachite green or rhodamine B [as described in Cutting and Roth (1973)]. The detection sensitivity of the staining method is considerably poorer than Coomassie Blue staining, with 80–160 ng of phosvitin, a protein containing roughly 100 phosphoserine residues, being detectable by the commercialized kit. The staining procedure is quite complex (involving seven different reagents) and alkaline hydrolysis requires heating gels to 65°C, which causes the gel matrix to hydrolyze and swell considerably. Since phosphotyrosine residues are not hydrolyzed by the alkaline treatment, proteins phosphorylated at this amino acid residue escape detection by the method. Dyes for the phosphate-selective fluorescence labeling in which a BODIPY dye is covalently attached to a

reactive imidazole group has been developed for the detection of pepsin phosphorylation. *See*, U.S. Pat. No. 5,512,486; Wang & Giese, "Phosphate-Specific Fluorescence Labeling of Pepsin by BO-IMI," *Anal. Biochem. 230*:329-332 (1995).

In addition to detecting phosphoproteins, two methods for the chemical derivatization and enrichment of phosphopeptides resulting in isolation of phosphopeptides from complex mixtures exist. *See, e.g. Goshe et al.*, "Phosphoprotein Isotope-Coded Affinity Tag Approach For Isolating and Quantitating Phosphopeptides in Proteome-Wide Analyses," *Anal. Chem. 73*:2578-2586 (2001). The first method involves oxidation of cysteine residues with performic acid, alkaline hydrolysis to induce β-elimination of phosphate groups from phosphoserine and phosphothreonine residues, addition of ethanedithiol, coupling of the resulting free sulfhydryl residues with biotin, purification of phosphoproteins by avidin affinity chromatography, proteolytic digestion of the eluted phosphoproteins, a second round of avidin purification and then analysis by mass spectrometry (Oda, Y., Nagasu, T., and Chait, B. *Nature Biotechnol. 19*:379 (2001)). The first method uses ß-elimination to remove phosphate groups that are replaced with a tag, as exemplified with biotinylated thiol groups wherein the peptides could then be isolated by chromatography on avidin resins. An alternative method requires proteolytic digestion of the sample, reduction and alkylation of cysteine residues, N-terminal and C-terminal protection of the peptides, formation of phosphoramidate adducts at phosphorylated residues by carbodiimide condensation with cystamine, capture of the phosphopeptides on glass beads coupled to iodoacetate, elution with trifluoroacetic acid and evaluation by mass spectrometry (Zhou *et al.*, "A Systematic Approach to the Analysis of Protein Phosphorylation," *Nat. Biotechnol. 19*:375-378 (2001). These methods are time consuming, require purified phosphopeptides, and are limiting in what can be isolated. Both procedures identified the monophosphorylated trypsin peptide fragment from the test protein β-casein, but both failed to detect the tetraphosphorylated peptide fragment.

Alternatively, a method for combining chemical modification and affinity purification has been shown for the characterization of serine and threonine phosphopeptides in proteins based on the conversion of phosphoserine and phosphothreonine residues to *S*-(2-mercaptoethyl)cysteinyl or ß-methyl-*S*-(2-mercaptoethyl)cysteinyl residues by β-elimination/1,2-ethanedithiol addition, followed by reversible biotinylation of the modified proteins. After trypsin digestion, the biotinylated peptides are affinity-isolated and enriched, followed by their subsequent structural characterization by liquid chromatography/tandem mass spectrometry (LC/MS/MS). See Adamczyk *et al.*, "Selective Analysis of

Phosphopeptides Within a Protein Mixture by Chemical Modification, Reversible Biotinylation and Mass Spectrometry," *Rapid. Commun. Mass Spectrom. 15*:1481-1488 (2001).

Fluorescence detection methods appear to offer the best solution to global protein quantitation in proteomics. However, currently, there is no satisfactory method for the specific and reversible fluorescent detection of gel-separated phosphoproteins from complex samples. Derivatization and fluorophore labeling of phosphoserine residues by blocking free sulfhydryl groups with iodoacetate or performate, alkaline β-elimination of the phosphate residue, addition of ethanedithiol, and reaction of the resulting free sulfhydryl group with 6-iodoacetamidofluorescein has been demonstrated in capillary electrophoresis using laser-induced fluorescence detection and similar reactions have been performed on protein microsequencing membranes. However, neither method has been shown to be suitable for detection of phosphoproteins directly in gels. One problem with the approach is that a delicate balance must be struck between the base and the ethanedithiol in order to achieve elimination of the phosphate group from the serine residue and addition of the ethanedithiol to the resulting dehydroalanine residue without hydrolysis of the peptide backbone.

Several instrument-based methods are also available for the determination of protein phosphorylation such as ^{31}P-NMR, mass spectrometry [*See, e.g.*, Resing & Ahn, "Protein Phosphorylation Analysis by Electrospray Ionization-Mass Spectrometery," *Methods Enzymol. 283*:29-44 (1997); Aebersold & Goodlett, "Mass Spectrometry in Proteomics," *Chem. Rev. 101*:269-295 (2001). Affolter, M., Watts, J., Krebs, D., and Aebersold, R. *Anal. Biochem.* 223:74 (1994); Liao, P., Leykam, J., Andrews, P., Gage, D., and Allison, J. *Anal. Biochem. 219*:9 (1994); Oda, Y., Huang, K., Cross, F., Cowburn, D., and Chait, B. *Proc. Natl. Acad. Sci. USA 96*:6591 (1999)) and protein sequencing. Mass spectrometry has been used to provide the molecular mass of an intact phosphorylated protein by comparing the mass of the unphosphorylated protein to that of the phosphorylated protein. *See, e.g.*, McLachlin & Chait, "Analysis of Phosphorylated Proteins and Peptides by Mass Spectrometry," *Current Opin. Chem. Biol. 5*:591-602 (2001). This is limiting in that researchers must have purified amounts of both proteins. While these procedures accurately characterize the phosphorylation status of proteins and peptides, they are unsuitable for high-throughput screening of phosphorylated substrates. The techniques are generally used after a phosphoprotein has been identified by autoradiography or immunoblotting with an anti-phosphotyrosine antibody. Though methods have recently been introduced to directly quantify the relative abundance of phosphoproteins in two different samples by mass spectrometry through culturing different cell populations in ^{15}N-enriched and ^{14}N-enriched medium, the linear dynamic range of such methods has explicitly been demonstrated over

only a 10-fold range. Ion suppression phenomena associated with mass spectrometry prevents stoichiometric comparison of different phosphoproteins by such techniques.

For analysis of the site(s) of phosphorylation on molecules, a more detailed analysis of the sites of phosphate attachment and stoichiometery often requires the examination of peptide fragments of the phosphoprotein of interest. Such fragments are usually generated by digestion of the phosphoprotein with proteases such as trypsin. However, mass spectroscopic analysis of proteolytic digests of proteins rarely provides full coverage of the protein sequence and regions of interests often go undetected. In addition, protein phosphorylation is often sub-stoichiometric, such that the phosphoproteins are present in lower abundance than other peptides from the protein of interest. Therefore, the identification and characterization of phosphoproteins would be improved greatly by highly selective methods of enriching phosphopeptides prior to analysis with mass spectrometry.

Currently, selective enrichment of phosphopeptides from complex mixtures is performed using immobilized metal affinity chromatography, known as IMAC. Using this technique, metal ions such as Fe^{3+} or Ga^{+3} are bound to a chelating support prior to the addition of a complex mixture of peptides or proteins. *See, e.g.*, Posewitz & Tempst, "Immobilized Gallium(III) Affinity Chromatography of Phosphopeptides," *Anal. Biochem. 71*:2883-2892 (1999). Phosphopeptides that bind to the column can be released using high pH or phosphate buffer, though the latter step usually requires a further desalting step before analysis with mass spectrometry. Resins with iminodiacetic acid and nitrilotriacetic acid chelators are known and are available commercially. *See, e.g.*, Neville *et al.*, "Evidence for Phosphorylation of Serine 753 in CFTR Using a Novel Metal-Ion Affinity Resin and Matrix-Assisted Laser Desorption Mass Spectrometry," *Protein Sci. 6*:2436-2445 (1997). However, there are several complications using current techniques, including loss of phosphopeptides that do not bind to the column (low affinity), difficulty in the subsequent elution of phosphorylated peptides, and background from non-phosphorylated peptides that have affinity for immobilized metal ions (low specificity).

Mass spectrometry–based detection of separated peptides and direct matrix-assisted laser desorption/ionization (MALDI) analysis of phosphopeptides bound to an IMAC support has been demonstrated. *See* Zhou *et al.*, "Detection and Sequencing of Phosphopeptides Affinity Bound to Immobilized Metal Ion Beads by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry," *J. Am. Soc. Mass. Spectrom. 11*:273-283 (2000). IMAC has also been coupled directly to mass spectrometry instruments on-line, or with superseding separation

techniques, such as HPLC and capillary electrophoresis (CE), for the detection and analysis of phosphopeptides.

The present invention overcomes the limitations of the current methods by utilizing a cationic transition metal and compound that comprises a metal-chelating moiety and a chemical moiety, typically a reactive group or label such as a fluorophore to detect phosphoproteins and phosphopeptides. There are a variety of chelating moieties that use poly-carboxylate binding sites to selectively bind monovalent and divalent metal cations, and these are often used in fluorescent calcium ion indicators. Examples of these indicators are, for example, quin-2, fura-2, indo-1 (U.S. Pat. No. 4,603,209); fluo-3 and rhod-2 (U.S. Pat. No. 5,049,673), and FURA RED™ (U.S. Pat. No. 4,849,362). A family of BAPTA-based indicators that are selective for calcium ions are described in HAUGLAND, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (9th edition, CD-ROM, September 2002). Examples of BAPTA-based metal-chelators are also described in U.S. Pat. Nos. 5,773,227; 5,453,517; 5,516,911; 5,501,980; 6,162,931 and 5,459,276.

Indicators of free calcium concentrations are based upon selective calcium binding to fluorescent dyes. Though it is well known that BAPTA compounds bind certain divalent cations, such as calcium, as analogs of the common EGTA chelator, the BAPTA compounds are also known to bind almost all inorganic polyvalent cations with an affinity that may be higher or lower than that of the compound for calcium ions. Their selectivity and utility for measuring calcium in biological cells results from the general absence or low abundance of these other polycations in living systems. The affinity and selectivity of the BAPTA-based indicators for polycations, including gallium and similar metals of utility to this invention, can be modified by shifts in pH, solvent composition, ionic strength and other experimental variables. This shift in cation selectivity and affinity is critical to all aspects of the disclosed invention, including both detection and isolation of phosphorylated targets.

The present invention overcomes the limitations and disadvantages of currently disclosed methods for identifying, isolating, analyzing and quantitating phosphorylated proteins and thus provides methods, compounds and compositions to alleviate long-felt needs for rapid and effective high-throughput methods for detecting and isolating phosphoproteins for further analysis. The present invention can accurately identify phosphopeptides and phosphoproteins comprising as few as one phosphate group and in a simple method that does not require multiple steps or pre-treatment of the sample. Importantly, the present invention is the first known method to provide a means for accurately identifying the phosphorylated proteome and allows for the quantitative identification of increased

and proteins. In this way, a particle could have many phosphate-binding compounds attached.

For detection purposes, A is typically a detectable label that is a dye including pigments, chromophores and fluorophores, a hapten, an enzyme, or a radioactive isotope, although an extensive assortment of other detectable labels that fall within the scope of this invention is known. For isolation purposes, A is typically a label or a reactive group that is bound to a polymer such as agarose or a microsphere. The polymer, in combination with the metal chelating moiety and a metal ion is selected to form a soluble or insoluble ternary complex with the phosphorylated target molecule. Such ternary complexes are particularly useful for the selective isolation of phosphorylated targets from complex mixtures or as components of various detection schemes. In a further aspect of the invention, A is a chemical moiety that alters the solubility of the ternary complex or alternatively comprises an amine reactive group used to form a covalent bond with an amine containing molecule including polymers and phosphate target molecules.

The "binding solution" of the invention (which we define to include true solutions, suspensions, emulsions, dispersions and immobilized variants) of the present invention comprises a phosphate-binding compound having the formula (A)m(L)n(B), a salt comprising selected metal ions, and an acid. The preferred salt and metal ion composition and concentration of the binding solution or suspension will depend to some extent on the metal-chelating moiety of the compound. A particularly useful binding solution is the combination of a BAPTA–based chelating moiety of the phosphate-binding compound and a gallium salt. Unexpectedly, we have determined that trivalent gallium ions simultaneously bind BAPTA moieties and phosphorylated target molecules to form a ternary complex with a useful affinity only in the presence of a moderately acidic environment. However, other metal chelating moieties such as DTPA, IDA and phenanthroline have been shown to simulateously bind gallium trivalent ions and phosphate groups. Thus, one requirement of the binding solution is the presence of an acid, wherein the binding solution preferably has a pH of about 3 to about 6; typically the pH is about 3 to about 4. The nature of the acid used to obtain this pH appears to be irrelevant; however, a phosphoric acid, phosphonic acid or polyphosphoric acid should not be used to obtain this pH, as they could reduce the stability of the ternary complex. Typically, the phosphate-binding compound is free in the binding solution or suspension; however, the phosphate-binding compound can be immobilized on a solid or semi-solid matrix such that when the metal ion and acid are added a binding solution is formed and a ternary complex of the invention is subsequently formed if a phosphorylated target is present in a sample.

The methods of the invention comprise contacting a sample with a binding solution comprising the phosphate-binding compound, the metal ion and the acid, incubating the sample and the binding solution for sufficient time to allow the compound of the binding solution to associate with said phosphorylated target molecule whereby said phosphorylated target molecule forms a ternary complex.

Typically, for detection purposes, the resulting ternary complex that comprises the compound is illuminated to measure a detectable optical property of the chemical moiety A, whereby the presence of the phosphorylated target molecules is detected. The phosphorylated target molecules can be detected in solution or when immobilized on a solid or semi-solid matrix. The compositions and methods of this invention can be used to detect phosphorylated target molecules present in a complex sample of phosphorylated and nonphosphorylated target molecules or to detect a change in the number of phosphate groups on a target molecule. Differences in the degree of phosphorylation can be due to intrinsic differences in the degree of phosphorylation of the biopolymer, which can cause differences in folding of proteins, or to an *in vivo* process such as a disease state or in conjunction with an *in vitro* assay to identify specific kinases and phosphatases.

Alternatively, when the method is utilized to selectively isolate phosphorylated target molecules from solution, visualizing the complex may not be necessary. To isolate the phosphorylated target molecules, the ternary complex can be precipitated, immobilized, separated by a chromatographic or electrophoretic technique or remain in solution. In some cases, organic extraction can be used to separate the metal chelating moiety from the phosphorylated target molecule. When the ternary complex is precipitated or otherwise immobilized, the phosphorylated target molecules can be separated from the nonphosphorylated target molecules and other components of the sample by affinity chromatography, such as by simple washing with an aqueous, organic or mixed aqueous/organic wash solution. In some cases, it is advantageous to further analyze the extracted phosphorylated target molecules while they are still immobilized on a matrix. Isolation of phosphorylated target molecules is useful for further analysis of the target molecules, such as by liquid chromatography/mass spectrography, an electrophoretic separation technique, by detection of the bound target molecules by an antibody to any part of the target molecule or by a variety of other techniques. In particular, isolation of the phosphorylated target molecules simplifies the subsequent analysis of the sample by removing interfering components of the original sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Shows the selective detection of a phosphorylated target molecule (ovalbumin) (1A) in a polyacrylamide gel using a binding solution comprising gallium chloride and Compound 2 compared to the same gel post-stained with a total protein stain (1B) (SYPRO® Ruby protein gel stain) (*See*, Example 2). The protein mixture was loaded at ca. 500 µg and contained nine total proteins, one of which was a phosphoprotein (ovalbumin) that contains two phosphate groups. The figure demonstrates selective detection of ovalbumin against a background of very low or no staining of eight proteins known to be non-phosphorylated.

Figure 2: Shows the selective detection of a phosphorylated target molecule (ovalbumin) (2A) on a PVDF membrane using a binding solution comprising gallium chloride and Compound 1 compared to the same membrane post-stained with a total protein stain (2B) (SYPRO® Ruby protein blot stain) (*See*, Example 8). The figure demonstrates selective detection of ovalbumin against a background of very low or no staining of five non-phosphorylated proteins.

Figure 3: Shows the sensitivity and linear dynamic range of detecting phosphorylated proteins in a gel using a binding solution comprising gallium chloride and Compound 2 (*See*, Example 2); (3A) is a comparison of five proteins with different ratios of phosphate groups and (3B) compares pepsin to bovine serum albumin (BSA). Proteins were loaded in two-fold dilution series on SDS polyacrylamide mini-gels from 2 ng – 1000 ng; each protein sample was done in series in four replicate gels. The phosphoproteins were α-casein (7 or 8 phosphates); dephosphorylated α-casein (1 or 2 phosphates); β-casein (5 phosphates); ovalbumin (2 phosphates) and pepsin (1 phosphate). BSA contains no phosphates and was used as a negative control. The results demonstrate that the methods and binding solution of the present invention can detect as little as 1–2 ng of a pentaphosphorylated protein (β-casein), and 8 ng of a monophosphorylated protein (pepsin).

Figure 4: Shows the detection of protein phosphatase activity wherein α-casein and pepsin were used as a phosphatase substrate. The gels were incubated with a binding solution comprising gallium chloride and Compound 2 to demonstrate a reduction in phosphate groups, compared to a control, after the substrates were incubated with a protein phosphatase, *See* example 6.

Figure 5: Shows the isolation of phosphopeptides (pT/pY and RII; MWs 1670 and 2193) (Panel B and C) from a solution containing non-phosphorylated peptides (angiotensin I and II, MWs 1297 and 1046) (Panel A) when the solution of peptides was incubated with a binding solution comprising gallium chloride and Compound 5. The mixture was incubated for 1 hour and centrifuged for 5 minutes. The resulting supernatants (bottom spectra in all panels) and pellet precipitates (top spectra in all panels) were analyzed by MALDI-TOF mass spectrometry. Panel A shows the non-phosphorylated peptides exclusively in the supernatants, while figures B and C show the two phosphopeptides of greater than 95% purity in the pellets.

Figure 6: Shows the analysis of phosphorylated peptides (α-casein) eluted from an affinity chromatography matrix column containing Compound 13 or Compound 14 that had been charged with gallium ions. Panel A shows differential MALDI-TOF MS analysis of purified α-casein phosphoserine peptides after dephosphorylation (left peaks in pairs) and subsequent derivatization with methylamine (right peaks in pairs). Results show that all three peptides are phosphoserine derivatives by methylamine addition. A and B of Panel A were monophosphorylated (+31 amu for methylamine) and C was triphosphorylated (+93 amu for 3 methylamines). Panel B shows a MALDI-TOF MS profile of eluted phosphopeptides from a BAPTA-agarose (Compound 13 or Compound 14) column versus commercially available metal affinity columns (Pierce Chemical Co., -.). Under the conditions used, the BAPTA-agarose column shows all expected phosphopeptides (arrows) purified from a complex peptide mix. Panel C shows the Control peptide (MW = 1870) with one phosphothreonine and one phosphotyrosine residue after treatment with strong base (-98 amu) and methylamine. Results show elimination of a single phosphate only (-98 amu from threonine) with no subsequent addition of methylamine (+32 amu), confirming a single phosphothreonine residue. Phosphotyrosine is determined by a lack of modification under these elimination conditions.

Figure 7: Shows the detection of a phosphoprotein (β-casein) on a HydroGel microarray (Perkin Elmer, Foster City, CA) when the microarray was incubated with a binding solution comprising Compound 2 and gallium chloride, *see* Example 18. The protein was loaded in a two-fold dilution series from 166 pg – 0.324 pg on the microarray. The results show the detection of 0.65 pg of a pentaphosphorylated protein on a HydroGel microarray.

of the Y-intercept values when plotted against the protein concentration resulting in

phosphoproteins with a ratio value 50–100 times greater than that of the non-phosphorylated proteins.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such may vary. It must be noted that, as used in this specification and the appended claims, the singular form "a", "an" and "the" includes plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a phosphorylated protein" includes a plurality of proteins and reference to "a compound" includes a plurality of compounds and the like.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. The following terms are defined for purposes of the invention as described herein.

The term "affinity" as used herein refers to the strength of the binding interaction of two molecules, such as a metal-chelating compound and a metal ion.

The term "alkyl" as used herein refers to a straight, branched, or cyclic hydrocarbon chain containing between one and twenty carbon atoms, typically between about one and about ten carbon atoms (e.g., methyl, ethyl, n-propyl, iso-propyl, cyclopropyl, n-butyl, iso-butyl, tert-butyl, cyclobutyl, adamantyl, noradamantyl, and the like) and attached to the compound by a carbon atom. Straight, branched or cyclic hydrocarbon chains having eight or fewer carbon atoms will also be referred to herein as "lower alkyl." The hydrocarbon chains may be saturated or include one or more degrees of unsaturation, i.e., one or more double or triple bonds (e.g., vinyl, propargyl, allyl, 2-buten-1-yl, 2-cyclopenten-1-yl, 1,3-cyclohexadien-1-yl, 3-cyclohexen-1-yl, 2-cyclopenten-1-yl, 1,3-cyclohexadien-1-yl, 3-cyclohexen-1-yl and the like). Alkyl groups containing double bonds will also be referred to herein as "alkenes" and alkyl groups containing triple bonds will be referred to herein as "alkynes". However, as used in context with respect to cyclic alkyl groups, the combinations of double and/or triple bonds do not include those bonding arrangements that render the cyclic hydrocarbon chain aromatic. Hydrocarbon chains having one or more noncarbon atoms (i.e., heteroatoms such

as N, S, O, P) in the chain will also be referred to herein as heteroalkyl. "Alkyl" further includes one or more substitutions at one or more carbon atoms of the hydrocarbon fragment or radical. Such substitutions include, but are not limited to: aryl; heteroaryl; halogen (F, Cl, Br, I or to form, e.g., trifluoromethyl, $-CF_3$); nitro ($-NO_2$); cyano (–CN); hydroxyl (also referred to herein as "hydroxy"), alkoxyl (also referred herein as alkoxy) or aryloxyl (also referred to herein as "aryloxy," –OR); thio or mercapto, alkyl, or arylthio (–SR); amino, alkylamino, arylamino, dialkyl– or diarylamino, or arylalkylamino (–NRR'); aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbonyl, diarylaminocarbonyl or arylalkylaminocarbonyl (–C(O)NRR'); carboxyl, or alkyl– or aryloxycarbonyl (–C(O)OR); aldehyde; aryl– or alkylcarbonyl (RC(=O)–); iminyl, or aryl– or alkyliminyl (–C(=NR)R'); where R and R' independently are hydrogen, aryl or lower alkyl as defined herein. Substituents that include one or more heteroatoms (i.e., heterocycle, heteroaryl, and heteroaralkyl) are defined by analogy to the above-described terms. For example, the term "heteroaryloxy" refers to the group –OR, where R is a heterocycle, as defined below.

The term "amino" or "amine group" refers to the group –NR'R" (or NRR'R") where R, R' and R" are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, aryl, substituted aryl, aryl alkyl, substituted aryl alkyl, heteroaryl, and substituted heteroaryl. A substituted amine being an amine group wherein R' or R" is other than hydrogen. In a primary amino group, both R' and R" are hydrogen, whereas in a secondary amino group, either, but not both, R' or R" is hydrogen. In addition, the terms "amine" and "amino" can include protonated and quaternized versions of nitrogen, comprising the group –NRR'R" and its biologically compatible anionic counterions.

The term "aryl" as used herein refers to cyclic aromatic carbon chains having twenty or fewer carbon atoms, e.g., phenyl, naphthyl, biphenyl, and anthracenyl. One or more carbon atoms of the aryl group may also be substituted with, e.g., alkyl; aryl; heteroaryl; a halogen; nitro; cyano; hydroxyl, alkoxyl or aryloxyl; thio or mercapto, alkyl-, or arylthio; amino, alkylamino, arylamino, dialkyl-, diaryl-, or arylalkylamino; aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbonyl, diarylaminocarbonyl, or arylalkylaminocarbonyl; carboxyl, or alkyl- or aryloxycarbonyl; aldehyde; aryl- or alkylcarbonyl; iminyl, or aryl- or alkyliminyl; sulfo; alkyl- or alkylcarbonyl; iminyl, or aryl- or alkyliminyl; sulfo; alkyl- or arylsufonyl; hydroximinyl, or aryl- or alkoximinyl. In addition, two or more alkyl or heteroalkyl substituents of an aryl group may be combined to form fused aryl–alkyl or aryl–heteroalkyl ring systems (e.g., tetrahydronaphthyl). Substituents including heterocyclic groups (e.g., heteroaryloxy, and heteroaralkylthio) are defined by analogy to the above-described terms.

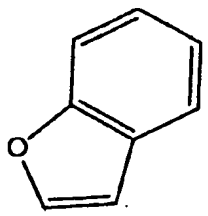
The term "aryloxy" refers to the group aryl–O– or heteroaryl-O–.

The term "arylalkyl" as used herein refers to an aryl group that is joined to a parent structure by an alkyl group as described above, e.g., benzyl, α-methylbenzyl, phenethyl, and the like.

The term "aqueous solution" as used herein refers to a solution that is predominantly water and retains the solution characteristics of water. Where the aqueous solution contains solvents in addition to water, water is typically the predominant solvent.

The term "BAPTA" as used herein refers to a metal-chelating compound that is 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid or its analogs, derivatives, ring-fused variants and conjugates, and all metallic and nonmetallic salts, partial salts and hydrates thereof, including any corresponding compounds disclosed in US Patent Nos. 4,603,209; 4,849,362; 5,049,673; 5,453,517; 5,459,276; 5,516,911; 5,501,980; 6,162,931 and 5,773,227 (*supra*). When used generically, "BAPTA" refers to two benzene rings that are joined by a C_1–C_3 hydrocarbon bridge terminated by oxygen atoms, including methylenedioxy (–OCH_2O–), ethylenedioxy (–OCH_2CH_2O–) or propylenedioxy (–$OCH_2CH_2CH_2O$–) bridging groups, where each benzene ring is optionally substituted by one or more substituents that adjust the metal ion–binding affinity, solubility, chemical reactivity, spectral properties or other physical properties of the compound. In a preferred embodiment of the present invention "BAPTA" is covalently attached to a chemical moiety A that, in combination with an appropriate trivalent metal ion and an acid, permits detection or isolation of phosphorylated target molecules as a ternary complex. BAPTA derivatives additionally include compounds in which the benzene rings of the BAPTA structure are substituted by or fused to additional aromatic, or heteroaromatic rings.

The term "benzofuran" as used herein refers to a dye that is a heterocycle or derivative thereof generally having the structure below.



acidity or basicity of the solution against addition or depletion of chemical substances.

Typically, the detectable response is an optical response resulting in a change in the wavelength distribution patterns or intensity of absorbance or fluorescence or a change in light scatter, fluorescence lifetime, fluorescence polarization, or a combination of these

parameters. Alternatively, the detectable response is an occurrence of a signal wherein the dye is inherently fluorescent and does not produce a change in signal upon binding to a metal ion or phosphorylated target molecule. Alternatively, the detectable response is the result of a signal, such as color, fluorescence, radioactivity or another physical property of the detectable label becoming spatially localized in a subset of a sample such as in a gel, on a blot, or an array, in a well of a micoplate, in a microfluidic chamber, or on a microparticle as the result of formation of a ternary complex of the invention that comprises a phosphorylated target molecule.

The term "directly detectable" as used herein refers to the presence of a detectable label or the signal generated from a detectable label that is immediately detectable by observation, instrumentation, or film without requiring chemical modifications or additional substances. For example, a fluorophore produces a directly detectable response.

The term "DTPA" as used herein referes to a metal chelating moiety diethylene triamine pentaacetic acid or derivatives thereof and any corresponding moieties disclosed in US Patent Nos. 4,978,763 and 4,647,447. DTPA is represented by the formula $(CH_2CO_2R^{13})_ZN[(CH_2)_SN(CH_2CO_2R^{13})]_T(CH_2)_SN(CH_2CO_2R^{13})_Z$ wherein the linker is attached to a methine carbon or nitrogen atom and Z is 1 or 2, S is 1 to 5, T is 0 to 4 and R^{13} is hydrogen or a salt.

The term "dye" as used herein refers to a compound that emits light to produce an observable detectable signal. "Dye" includes fluorescent and nonfluorescent compounds that include without limitations pigments, fluorophores, chemiluminescent compounds, luminescent compounds and chromophores. The term "fluorophore" as used herein refers to a composition that is inherently fluorescent or demonstrates a change in fluorescence upon binding to a biological compound or metal ion, or metabolism by an enzyme, i.e., fluorogenic. Fluorophores may be substituted to alter the solubility, spectral properties or physical properties of the fluorophore. Fluorophores of the present invention are not sulfonated. Numerous fluorophores are known to those skilled in the art and include, but are not limited to benzofurans, quinolines, quinazolinones, indoles, benzazoles, borapolyazaindacenes and xanthenes, with the latter including fluoresceins, rhodamines and rhodols as well as other fluorophores described in RICHARD P. HAUGLAND, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (9th edition, including the CD-ROM, September 2002).

The term "enzyme" as used herein refers to a protein molecule produced by living organisms, or through chemical modification of a natural protein molecule, that catalyzes chemical reaction of other substances without itself being destroyed or altered upon completion of the reactions. Examples of other substances, include, but are not limited to chemiluminescent, chromogenic and fluorogenic substances or protein-based substrates.

The term "halogen" as used herein refers to the substituents fluoro, bromo, chloro, and iodo and compounds containing combinations of these halogens and multiple copies of any halogen.

The term "heteroaryl" as used herein refers to an aryl group as defined above in which one or more carbon atoms have been replaced by a non-carbon atom, especially nitrogen, oxygen, or sulfur. For example, but not as a limitation, such groups include furyl, tetrahydrofuryl, pyrrolyl, pyrrolidinyl, thienyl, tetrahydrothienyl, oxazolyl, isoxazolyl, triazolyl, thiazolyl, isothiazolyl, pyrazolyl, pyrazolidinyl, oxadiazolyl, thiadiazolyl, imidazolyl, imidazolinyl, pyridyl, pyridaziyl, triazinyl, piperidinyl, morpholinyl, thiomorpholinyl, pyrazinyl, piperainyl, pyrimidinyl, naphthyridinyl, benzofuranyl, benzothienyl, indolyl, indolinyl, indolizinyl, indazolyl, quinolizinyl, qunolinyl, isoquinolinyl, cinnolinyl, phthalazihyl, quinazolinyl, quinoxalinyl, pteridinyl, quinuclidinyl, carbazolyl, acridinyl, phenazinyl, phenothizinyl, phenoxazinyl, purinyl, benzimidazolyl and benzthiazolyl and their aromatic ring–fused analogs. Many fluorophores are comprised of heteroaryl groups and include, without limitations, xanthenes, oxazines, benzazolium derivatives (including cyanines and carbocyanines), borapolyazaindacenes, benzofurans, indoles and quinazolones.

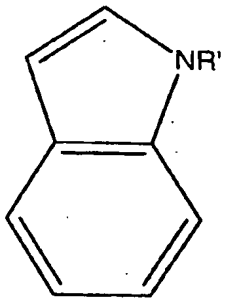
The above heterocyclic groups may further include one or more substituents at one or more carbon and/or non-carbon atoms of the heteroaryl group, e.g., alkyl; aryl; heterocycle; halogen; nitro; cyano; hydroxyl, alkoxyl or aryloxyl; thio or mercapto, alkyl- or arylthio; amino, alkyl-, aryl-, dialkyl-, diaryl-, or arylalkylamino; aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbonyl, diarylaminocarbonyl or arylalkylaminocarbonyl; carboxyl, or alkyl- or aryloxycarbonyl; aldehyde; aryl- or alkylcarbonyl; iminyl, or aryl- or alkyliminyl; sulfo; alkyl- or arylsulfonyl; hydroximinyl, or aryl- or alkoximinyl. In addition, two or more alkyl substituents may be combined to form fused heterocycle-alkyl ring systems. Substituents including heterocyclic groups (e.g., heteroaryloxy, and heteroaralkylthio) are defined by analogy to the above-described terms.

The term "heterocyclealkyl" as used herein refers to a heterocycle group that is joined to a parent structure by one or more alkyl groups as described above, e.g., 2-piperidylmethyl,

and the like. The term "heterocycloalkyl" refers to a heteroaryl group that is joined to a parent structure by one or more alkyl groups as described above, e.g., 2-thienylmethyl, and the like.

The term "IDA" as used herein refers to imidodiacetic acid metal chelating moieties having the formula $-N(CH_2CO_2R^{13})_2$ wherein R^{13} is hydrogen or a salt and the linker is attached to the nitrogen atom provided that the linker is not a single covalent bond attached to an aromoatic ring of a fluorophore.

The term "indole" as used herein refers to a compound or derivative thereof generally having the formula wherein R' is as defined above for amine group.



A linker at any of the aromatic carbon atoms substitutes the indole compound and preferably the linker attaches the indole to a BAPTA and is a member of the family of Ca^{2+}-ion indicators typically referred to as "indo" indicators (US Patent No. 4,603,209). Alternatively, the benzene ring of the indole forms one of the aromatic rings of the BAPTA chelating moiety. The indole may also be further substituted by substituents that adjust the solubility, metal ion affinity or specificity, spectral properties or other physical properties of the indole.

The term "isolated" as used herein with reference to the subject peptides, proteins and protein complexes, refers to a preparation of a peptide, protein or protein ternary complex that is essentially free from contaminating nonphosphorylated peptides, proteins or other associated target molecules that normally would be present in association with the peptide, protein or complex, e.g., in a cellular mixture or milieu in which the protein or complex is found endogenously. In addition, in some embodiments, "isolated" also refers to the further separation from reagents of the invention used to isolate the peptide, protein or complex from cellular mixture. Thus, an isolated protein or protein complex is separated (isolated) from other components of the sample and optionally from the phosphate-binding compounds of the invention (including polymeric matrices) that normally would "contaminate" or interfere with the study or further processing of the complex in isolation, such as by mass

spectrometry. The term "isolated" can also refer to phosphorylated target molecules that are spatially or temporally separated from each other such as by different physical locations on a gel or array or by having different passage times through a detector such as in a column or capillary.

The term "kit" as used herein refers to a packaged set of related components, typically one or more compounds or compositions, optionally comprising buffers, separation media, standards, software and other components.

The term "label" as used herein refers to a detectable moiety that is used to facilitate detection and isolation of phosphorylated target molecules in combination with the metal-chelating moieties of the present invention. Illustrative labels include labels that can be directly observed or measured or indirectly observed or measured such as fluorophores, radioactive and enzyme reporters labels Patton, W. (2002) "Detection technologies in proteome analysis." *J.Chromatography B: Biomedical Applications* 771: 3-31. Patton, W. (2000) "A Thousand Points of Light; The Application of Fluorescence Detection Technologies to Two-Dimensional Gel Electrophoresis and Proteomics". *Electrophoresis* 21, 1123-1144. Such labels include, but are not limited to, radiolabels that can be measured with radiation-counting devices; pigments, dyes or other chromogens that can be visually observed or measured with a spectrophotometer; spin labels that can be measured with a spin label analyzer; and fluorescent labels (fluorophores), where the output signal is generated by the excitation of a suitable molecular adduct and that can be visualized by excitation with light that is absorbed by the dye or can be measured with standard fluorometers or imaging systems, for example or metal particles, e.g. gold or silver particles or metallic bar codes that can be detected by their optical or light-scattering properties. The label can be a chemiluminescent substance, where the output signal is generated by chemical modification of the signal compound; a metal-containing substance; or an enzyme, where there occurs an enzyme-dependent secondary generation of signal, such as the formation of a colored product from a colorless substrate. The term label can also refer to a "tag", hapten or other ligand that can bind selectively to a labeled molecule such that the labeled molecule, when added subsequently, is used to generate a detectable signal. For example, one can use biotin as a tag and then use an avidin or streptavidin conjugate of horseradish peroxidase (HRP) to bind to the tag, and then use a chromogenic substrate (e.g., tetramethylbenzidine) or a fluorogenic substrate such as Amplex® Red reagent (Molecular Probes, Inc.) to detect the presence of HRP. Numerous labels and tags and methods for their selective detection are known by those of skill in the art and include, but are not limited to, particles, fluorophores, haptens, enzymes and their chromogenic,

fluorogenic and chemiluminescent substrates and other labels that are described in the MOLECULAR PROBES HANDBOOK, *supra.*

The term "Linker" or "L" as used herein refers to a single covalent bond or a series of stable covalent bonds incorporating 1–30 nonhydrogen atoms selected from the group consisting of C, N, O, S and P that covalently attach the label to the metal-chelating moiety of the labeling compounds.

The term "metal-chelator" or "metal-chelating moiety" as used herein refers to a chemical moiety that combines with a metal ion to form a chelate ring structure. For the purposes of the present invention the metal chelator has affinity for a metal ion that has simultaneous affinity for the metal chelator and a phosphate target molecule in a moderately acidic environment. Examples of metal-chelating moieties are BAPTA, IDA, DTPA and phenanthroline. The metal-chelators are optionally substituted by substituents that adjust the ion-binding affinity, solubility, spectral properties or other physical properties of the compound provided that the metal-chelator is not sulfonated.

The term "metal ion" as used herein refers to any trivalent metal ion that has simultaneous affinity for a phosphate group of a target molecule and a metal-chelating compound of the invention at pH 3 to 6 and that can be used to form a ternary complex of the phosphate-binding compound and the phosphorylated target molecule. Such metal ions include, without limitation, Al^{3+}, Fe^{3+} and Ga^{3+}. For purposes of the present invention, the metal ion must have simultaneous affinity for both the metal-chelating moiety and phosphate groups of the target molecule and, as such, confers affinity to the metal-chelating moiety for the phosphate groups of the taraget molecule that would not be present without the metal ion.

The term "phosphate-binding compound" or "binding compound" as used herein refers to a compound having the formula (A)m(L)n(B)n wherein A is a chemical moiety, L is a linker, B is metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4. These compounds effectively attach a label to a phosphorylated target molecule when the metal-chelating moiety indirectly binds phosphate groups on the target molecule.

The terms "phosphorylated target molecule" or "phosphate target molecule" as used herein refers to a molecule possessing one or more phosphate or phosphate analog moieties each attached to such molecule by a single ester bond or inorganic phosphate. . Phosphate analogs include, without limitation, thiophosphate, boraophosphate, phosphoarimide, H-phosphonate, alkylphosphonate, phosphorothioate, phosphorodithioate and

phosphorofluoridate. Phosphorylated target molecules include, but are not limited to, phosphoproteins, phosphopeptides, phospholipids, phosphoglycans, phosphocarbohydrates, phosphoamino acids, pyrophosphate and inorganic phosphate and their phosphate analogs. Most known phosphate compounds, and subsequently the phosphorylated target molecules, can be categorized into one of three groups; 1) individual phosphate groups (e.g., inorganic phosphate or a phosphate group (PO_3) on a protein or peptide); 2) multiple-linked phosphate group (e.g., pyrophosphate or a nucleotide such as ATP); or 3) bridging phosphate group (i.e., nucleic acids). For the purposes of the present invention, phosphorylated target molecules do not include molecules in the third group, e.g., DNA or RNA. Typically, phosphoproteins and phosphopeptides are phosphorylated post-translationally on the tyrosine, serine or threonine amino acid residues. Other phosphorylated amino acid residues in peptides and proteins include 1-phospho-histidine, 3-phospho-histidine, phospho-aspartic acid, phospho-glutamic acid and less commonly N^{ε}-phospho-lysine, N^{ω}-phospho-arginine and phospho-cysteine (Kaufmann, et al (2001) Proteomics 1: 194-199; Yan, J., Paxker, N., Gooley, A. and Williams, K. (1998) J. Chromatograph. A 808: 23-41). Thus, a phosphorylated protein or peptide typically comprises at least one of these amino acid residues. Phosphorylated target molecules also include phosphorylated proteins that incorporate other non-peptide regions such as lipids or carbohydrates, e.g., lipoproteins and lipopolysaccharides. In addition, the lipid or carbohydrate residues of the proteins can be phosphorylated instead or in combination with the tyrosine, serine or threonine amino acid residues of the proteins and peptides such as a phosphomannose-modified or N-acetylglucosamine-1-phosphate modified protein. Other modifications include a pyridoxal phosphate Schiff base to the epsilon-amino group of lysine, and an O-pantetheine phosphorylation of serine residue. The gamma phosphate of nucleotide triphosphates is also detectable using the methods of this invention, making photolabeled proteins and peptides detectable by this procedure. For the purposes of the present invention phosphorylated target molecules include phosphorylated lipids and carbohydrates.

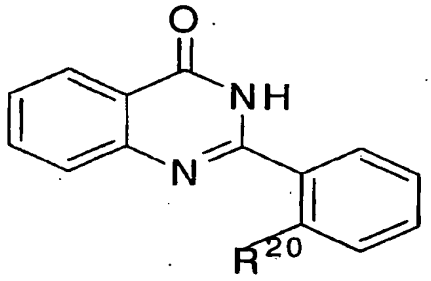
The terms "protein" and "polypeptide" are used herein in a generic sense to include polymers of amino acid residues of any length. The term "peptide" is used herein to refer to polypeptides having less than 100 amino acid residues, typically less than 15 amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The peptide or protein may be further conjugated to or complexed with other moieties such as dyes, haptens, radioactive isotopes,

natural and synthetic polymers (including microspheres), glass, metals and metallic particles, proteins and nucleic acids.

The term "sample" as used herein refers to any material that may contain phosphorylated target molecules, natural or synthetic, as defined above, or contains components that directly interact with phosphate or phosphorylated target molecules, such as enzymes. Typically, the sample comprises purified or semi-purified phosphorylated target molecules and endogenous host cell proteins. The phosphorylated target molecules can be made synthetically or obtained in a purified or semi-purified form from cells (eukaryotic and prokaryotic, without limitation) cell extracts, cell homogenates, subcellular components as natural or recombinant molecules. Alternatively, phosphorylated target molecules can be obtained from tissue homogenate, bodily and other biological fluids, or synthesized proteins, all of which comprise a sample in the present invention. The sample may be in an aqueous or mostly aqueous solution, a viable cell culture or immobilized on a solid or semi solid surface such as a polymer gel, a membrane, a microparticle, an optical fiber or on a microarray. In addition "sample" as use herein also refers to substrates for kinases or phosphatases or molecules that bind phosphorylated target molecules that may or may not be phosphorylated. In this way the sample comprises components that interact with phosphate and phosphorylated target molecules, particularly including antibodies to either the phosphorylated target molecules or to other regions of the target molecule.

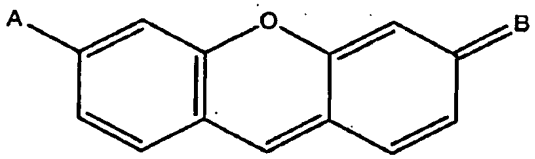
The term "ternary complex" as used herein refers to a composition that simultaneously comprises a phosphate-binding compound, a trivalent metal ion of the present invention and a phosphate target molecule, wherein the metal ions simultaneously have affinity for both the metal-chelating moiety of the compound and the phosphate group on the molecule, and wherein the metal ion forms a bridge between the two molecules. Unless limited by the context of their use, the terms "binding" and "complex formation" in this invention mean the process of formation of this ternary complex.

The term "quinazolinone" as used herein refers to a compound or derivative thereof generally having the structure:



A linker at any of the aromatic carbon atoms substitutes the compound and the linker attaches the compound to a metal-chelating moiety. Alternatively, either of the aromatic rings of the compound also forms an aromatic ring of the BAPTA chelating moiety, wherein no linker is present to attach the compound to the metal-chelating moiety. The compound may also be further substituted by substituents that adjust the solubility, metal ion affinity or specificity, spectral properties or other physical properties of the compound. A particularly advantageous substitution is a hydroxyl (OH) group at R^{20}.

The term "xanthene" as used herein refers to a compound or derivative thereof generally having the formula wherein A is amino or substituted amino or OH and B is O, amino or substituted amino. Rhodol compounds are represented when A is amino or substituted amino and B is O, rhodamine compounds are represented when both A and B are independently amino or substituted amino and fluorescein compounds are represented when A is OH and B is O.



By substituted amino is meant derivatives of nitrogen in which the substituents are independently C_1 to C_8 alkyl, aryl or heteroaryl, including derivatives in which the nitrogen is linked to positions ortho to the nitrogen by 5- or 6-membered aliphatic rings.

Xanthenes may be further substituted at any of the aromatic carbon positions by substituents well known in the art, including but not limited, to the substituents disclosed in US patent Nos. 5,049,673; 5,453,517; 6,130,101 and 6,162,931.

II. COMPOSITIONS AND METHODS OF USE

The present invention provides phosphate-binding compounds, a phosphate-binding solution and methods for selectively detecting and/or isolating phosphorylated target molecules. The phosphate-binding compounds of the present invention, when present in the binding solution, selectively bind to phosphorylated target molecules and permit detection and/or isolation of the target molecules. The binding solution comprises three critical components for binding phosphorylated target molecules: 1) phosphate-binding compounds having the formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4; 2) a salt comprising a metal ion and 3) an acid. The binding solution typically includes a buffering agent to maintain the acidic pH, which is ideally about pH 3 to about pH 6, and an organic solvent, wherein the use and solvent depends on the application and will be discussed below. The ternary complex that comprises the phosphate-binding compound, metal ion and phosphorylated target molecule is stable in an acidic environment but when the pH approaches neutral (pH 7) or basic (pH >7.0) the complex becomes increasingly unstable.

The binding solution is used to noncovalently attach a chemical moiety A, or a natural or synthetic substance when A is a reactive group, of the present invention to exposed phosphate groups on phosphorylated target molecules, wherein the chemical moiety A comprises the phosphate-binding compounds of the present invention. These bound target molecules can be subsequently detected using one of the detection methods described herein or isolated by a number of methods described below. The metal ions of the binding solution simultaneously have affinity for both phosphate groups and the metal-chelating moiety of the phosphate-binding compounds of the invention when in an acidic environment.

Thus, a method of the present invention for the binding of phosphorylated target molecules by a phosphate-binding compound comprises the following steps:

i) contacting the sample with a binding solution, and;

ii) incubating the sample and the binding solution for sufficient time to allow said compound to associate with said phosphorylated target molecules, whereby said phosphorylated target molecule is bound.

The methods of the present invention can be used in unlimited assay formats, provided that there is sufficient contact between the sample and the binding solution. Therefore, this method is intended to cover an unlimited number of assays, in any format, wherein the binding solution of the present invention has contact with an exposed phosphate group on a target molecule, regardless of the intent of the assay. Thus, the methods of the present invention contemplate, without limit, the identification of phosphorylated target molecules, identification of dephosphorylated molecules, identification of enzymes responsible for phosphorylation or dephosphorylation, directly or indirectly, identification of molecules that interact with phosphorylated target molecules and isolation of phosphorylated targetmolecules. Detection includes — where practical — quantitation, discrimination and subsequent analysis and identification of the phosphorylated target molecules, with the use of standards and controls, as appropriate.

In general, for ease of understanding the present invention, the components of the binding solution will first be described in detail, followed by the many and varied methods in which the phosphate-binding compounds and metal ions find uses, which is followed by exemplified methods of use and synthesis of certain novel compounds that are particularly advantageous for use with the methods of the present invention.

A. Components of the Phosphate-binding Compounds

The phosphate-binding compounds of the present invention have the formula (A)m(L)n(B), wherein A is a chemical moiety and L is a linker that covalently attaches the chemical moiety to the metal-chelating moiety (B). Typically the chemical moiety is a reactive group or a label that includes is a dye, a hapten or an enzyme. The metal-chelating moiety is dictated by metal ions that have affinity for phosphate and phosphate analog groups; such ions include Ga^{3+}, Fe^{3+} and Al^{3+}. It was found that for purposes of the present invention trivalent gallium ions when in a moderately acidic environment, e.g. between about pH 3 and about pH 6, have affinity for phosphate groups on target molecules and certain chelating groups such as BAPTA, IDA, DTPA and phenanthroline; BAPTA chelating moieties are the most preferred.

1. Chemical moieties A of the phosphate-binding compounds

A. Labels

The label of the phosphate-binding compound can be any label known to one skilled in the art and when the label is either covalently linked to a metal-chelating moiety or comprises

part of the metal-chelating moiety wherein no linker is present, forms a phosphate-binding compound of the present invention. Labels include, without limitation, a chromophore, a fluorophore, a fluorescent protein, a phosphorescent dye, a tandem dye (energy transfer pair), a microparticle, a polymer, a hapten, an enzyme and a radioisotope. Preferred labels include dyes, fluorescent proteins, haptens, and enzymes. The covalent linkage can be a single covalent bond or a combination of stable chemical bonds. The covalent linkage binding the label to the metal-chelating moiety is typically a single covalent bond, but can also be a substituted alkyl chain that incorporates 1–30 nonhydrogen atoms, or a substituted cycloalkyl, selected from the group consisting of C, N, O, S and P.

A dye of the present invention is any chemical moiety that exhibits an absorption maximum beyond 280 nm, that when part of a phosphate-binding compound retains its unique spectral properties to provide a detectable signal. The preferred dyes are fluorophores or chemiluminescence precursors that are directly detectable or that upon action of an additional reagent or reagents yield fluorescence or chemiluminescence.

Dyes of the present invention include, without limitation; a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a cyanine including any corresponding compounds in US Serial Nos. 09/968,401 and 09/969,853 and US Patent Nos. 6,403,807 and 6,348,599), a carbocyanine (including any corresponding compounds in US Serial No. 09/557,275 and US Patent Nos. 5,486,616; 5,268,486; 5,569,587; 5,569,766; 5,627,027 and 6,048,982), a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a borapolyazaindacene (including any corresponding compounds disclosed in US Patent Nos. 4,774,339; 5,187,288; 5,248,782; 5,274,113; and 5,433,896, *supra*), a xanthene (including any corresponding compounds disclosed in U.S. Patent No. 6,162,931; 6,130,101; 6,229,055; 6,339,392; 5,451,343 6,221,606; 6,358,684; 6,008,379; 6,111,116; 6,184,379; 6,017,712; 6,080,852; 5,847,162 and US serial No. 09/922,333) an oxazine or a benzoxazine, a carbazine (including any corresponding compounds disclosed in US Patent No. 4,810,636), a phenalenone, a coumarin (including an corresponding compounds disclosed in US Patent Nos. 5,696,157; 5,459,276; 5,501,980 and 5,830,912), a benzofuran (including any corresponding compounds disclosed in US Patent Nos. 4,603,209 and 4,849,362) and benzphenalenone (including any corresponding compounds disclosed in US Patent No. 4,812,409) and derivatives thereof. As used herein, oxazines include resorufins (including any corresponding compounds disclosed in US Patent No. 5,242,805), aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

Where the dye is a xanthene, the dye is optionally a fluorescein, a rhodol (including any corresponding compounds disclosed in US Patent Nos. 5,227,487 and 5,442,045), a rhodamine (including any corresponding compounds in US Patent Nos. 5,798,276 and 5,846,737). As used herein, rhodamine and rhodol dyes include, among other derivatives, compounds that comprise xanthenes with saturated or unsaturated "julolidine" rings. As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors (including any corresponding compounds disclosed in U.S. Patent No. 4,945,171).

Preferred dyes of the present invention include benzofurans, quinolines, quinazolinones, xanthenes, indoles, benzazoles and borapolyazaindacenes. Preferred xanthenes include julolidine-containing xanthenes, as well as fluoresceins, rhodols, rhodamines and rosamines. Xanthenes of this invention comprise both compounds substituted and unsubstituted on the carbon atom of the central ring of the xanthene by substituents typically found in the xanthene-based dyes such as phenyl and substituted-phenyl moieties. It is an important aspect of the current invention that none of the preferred fluorescent dyes are sulfonated.

Alternatively, the dye is a xanthene that is bound via an L that is a single covalent bond at the 9-position of the xanthene. Preferred xanthenes include derivatives of 3*H*-xanthen-6-ol-3-one attached at the 9-position, derivatives of 6-amino-3*H*-xanthen-3-one attached at the 9-position, or derivatives of 6-amino-3*H*-xanthen-3-imine attached at the 9-position.

Typically the dye contains one or more aromatic or heteroaromatic rings that are optionally substituted one or more times by a variety of substituents, including without limitation, halogen, nitro, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, aryl or heteroaryl ring system, benzo, or other substituents typically present on dyes known in the art.

In one aspect of the invention, the dye has an absorption maximum beyond 480 nm. In a particularly useful embodiment, the dye absorbs at or near 488 nm to 514 nm (particularly suitable for excitation by the output of the argon-ion laser excitation source) or near 546 nm (particularly suitable for excitation by a mercury arc lamp). As is the case for many dyes, they can also function as both chromophores and fluorophores, resulting in compounds that simultaneously act both as colorimetric and fluorescent labels for phosphorylated target molecules. Thus, the described fluorescent dyes are also the preferred chromophores of the present invention.

In addition to dyes, enzymes also find use as labels for the phosphate-binding compounds having the formula (A)m(L)n(B). Enzymes are desirable labels because amplification of the detectable signal can be obtained resulting in increased assay sensitivity. The enzyme itself does not produce a detectable response but functions to break down a substrate when it is contacted by an appropriate substrate such that the converted substrate produces a fluorescent, colorimetric or luminescent signal. Enzymes amplify the detectable signal because one enzyme on a labeling compound can result in multiple substrate molecules being converted to a detectable signal. This is advantageous where there is a low quantity of phosphorylated target molecules present in the sample or a fluorophore does not exist that will give comparable or stronger signal than the enzyme. Fluorophores are most preferred because they do not require additional assay steps that can lead to an unstable ternary complex. The enzyme substrate is selected to yield the preferred measurable product, e.g. color, fluorescence or chemiluminescence. Such substrates are extensively used in the art, many of which are described in the MOLECULAR PROBES HANDBOOK, *supra*.

A preferred colorimetric or fluorogenic substrate and enzyme combination uses oxidoreductases such as horseradish peroxidase (HRP) and a substrate such as 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC), which yield a distinguishing color (brown and red, respectively). Other colorimetric oxidoreductase substrates that yield detectable products include, but are not limited to: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *o*-phenylenediamine (OPD), 3,3',5,5'-tetramethylbenzidine (TMB), *o*-dianisidine, 5-aminosalicylic acid and 4-chloro-1-naphthol. Fluorogenic substrates include, but are not limited to, homovanillic acid or 4-hydroxy-3-methoxyphenylacetic acid, reduced phenoxazines and reduced benzothiazines, including Amplex® Red reagent and its variants (U.S. Pat. No. 4,384,042) and reduced dihydroxanthenes, including dihydrofluoresceins (U.S. Pat. No. 6,162,931) and dihydrorhodamines, including dihydrorhodamine 123. Peroxidase substrates that are tyramides (U.S. Pat. Nos. 5,196,306; 5,583,001 and 5,731,158) represent a unique class of peroxidase substrates in that they can be intrinsically detectable before action of the enzyme but are "fixed in place" by the action of a peroxidase in the process described as tyramide signal amplification (TSA). These substrates are extensively utilized to label targets in samples that are cells, tissues or arrays for their subsequent detection by microscopy, flow cytometry, optical scanning and fluorometry.

Another preferred colorimetric (and in some cases fluorogenic) substrate and enzyme combination uses a phosphatase enzyme such as an acid phosphatase or a recombinant

chromatography for isolation purposes. For detection purposes, an enzyme conjugate that has affinity for biotin is used, such as avidin-HRP. Subsequently a peroxidase substrate is

added to produce a detectable signal. For isolation purposes, a protein such as avidin that has affinity for biotin is conjugated to agarose beads. The biotin labeled metal-chelating moiety, after contacting a phosphorylated target molecule, is then incubated with the avidin beads, on a column or in solution, to separate and/or concentrate the phosphorylated target molecules. A preferred form of biotin is the desthiobiotin analog, which can be easily adsorbed and released from avidin-based affinity matrices. A preferred form of avidin for some applications is CaptAvidin biotin-binding protein (Molecular Probes), which permits facile release of biotinylated compounds.

Haptens also include, among other derivatives, hormones, naturally occurring and synthetic drugs, pollutants, allergens, affector molecules, growth factors, chemokines, cytokines, lymphokines, amino acids, peptides, chemical intermediates, nucleotides and the like.

Fluorescent proteins also find use as labels for the phosphate-binding compounds of the present invention. Examples of fluorescent proteins include green fluorescent protein (GFP) and the phycobiliproteins and the derivatives thereof. The fluorescent proteins, especially phycobiliproteins, are particularly useful for creating tandem dye–labeled labeling reagents or for indirect detection of hapten-labeled labeling compounds or phosphorylated target molecules that are immobilized on a matrix, such as a microsphere or an array. These tandem dyes comprise a fluorescent protein and a fluorophore for the purposes of obtaining a larger Stokes shift, wherein the emission spectra are farther shifted from the wavelength of the fluorescent protein's absorption spectra. This property is particularly advantageous for detecting a low quantity of a target molecule in a sample wherein the emitted fluorescent light is maximally optimized; in other words, little to none of the emitted light is reabsorbed by the fluorescent protein. For this to work, the fluorescent protein and fluorophore function as an energy transfer pair wherein the fluorescent protein emits at the wavelength that the acceptor fluorophore absorbs and the fluorophore then emits at a wavelength farther from the fluorescent proteins than could have been obtained with only the fluorescent protein. Alternatively, the fluorophore functions as the energy donor and the fluorescent protein is the energy acceptor. Particularly useful fluorescent proteins are the phycobiliproteins disclosed in US Patents 4,520,110; 4,859,582; 5,055,556 and the fluorophore bilin protein combinations disclosed in US Patent 4,542,104. Alternatively, two or more fluorophore dyes can function as an energy transfer pair wherein one fluorphore is a donor dye and the other is the acceptor dye (including any dye compounds disclosed in US Patent Nos. 6,358,684; 5,863,727; 6,372,445 and 5,656,554).

B. Reactive Groups

plastic such as microplate wells, polymers such as PVDF, nitrocellulose, polysaccharides in

sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

* Activated esters, as understood in the art, generally have the formula -COΩ, where Ω is a good leaving group (e.g. succinimidyloxy ($-OC_4H_4O_2$) sulfosuccinimidyloxy ($-OC_4H_3O_2-SO_3H$), -1-oxybenzotriazolyl ($-OC_6H_4N_3$); or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride $-OCOR^a$ or $-OCNR^aNHR^b$, where R^a and R^b, which may be the same or different, are C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, or C_1-C_6 alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).
** Acyl azides can also rearrange to isocyanates

2. Linkers

As described above, the chemical moiety A of the present invention is part of the phosphate-binding compounds, wherein they are either covalently attached to a metal-chelating moiety by a linker to form the compounds of the present invention or the label and the metal-chelating moiety share an aromatic ring, e.g., benzofuran and BAPTA. Thus, when the chemical moiety and chelating moiety share an aromatic ring no linker is present and n of the formula (A)m(L)n(B) is 0. A preferred embodiment is labeling compounds wherein no linker is present, however linkers as single covalent bonds are equally preferred.

When a linker is present, the linker typically incorporates 1–30 nonhydrogen atoms selected from the group consisting of C, N, O, S and P. The linker is typically a substituted alkyl or a substituted cycloalkyl. Alternatively, the fluorophore may be directly attached (where Linker is a single bond) to the metal-chelating moiety or the alkyl linker may contain a benzene ring or substituted benzene ring with substituents well known in the art for benzene rings. When the linker is not a single covalent bond, the linker may be any combination of stable chemical bonds, optionally including, single, double, triple or aromatic carbon–carbon bonds, as well as carbon–nitrogen bonds, nitrogen–nitrogen bonds, carbon–oxygen bonds, sulfur–sulfur bonds, carbon–sulfur bonds, phosphorus–oxygen bonds, phosphorus–nitrogen bonds, and nitrogen–platinum bonds. Preferrably the linker incorporates less than 20 nonhydrogen atoms and is composed of any combination of ether, thioether, thiourea, amine, ester, carboxamide, sulfonamide and hydrazide bonds and aromatic or heteroaromatic bonds. Typically the linker is a combination of single carbon–carbon bonds and carboxamide, sulfonamide, ether or thioether bonds. The bonds of the linker typically result in the following moieties that can be found in the linker: ether, thioether, carboxamide, thiourea, sulfonamide, urea, urethane, hydrazine, alkyl, aryl, heteroaryl, alkoxy, cycloalkyl and amine moieties. Selected examples of labeling compounds incorporate the following three (I, II and III) Linker formulas: Formula (I)

$-(CH_2)_eC(X)NH(CH_2)_e(NHC(X)(CH_2)_e)_d-$ and Formula (II)

-$((C_6R''_4)O)_d(CH_2)_e(C(X)NH(CH_2)_e)_g(NH)_dC(X)NH(C_6R''_4)(CH_2)_e$-, Formula (III) –$(NHC(X)(NH)_d(CH_2)_e(NH)_dC(X)(NH)_d(CH_2)_e(NHC(X)(CH_2)_e)_d)$-, wherein X is O or S, d is 0–1, e is 0–6, g is 1–4 and R" is independently H, halogen, alkoxy or alkyl. It is understood that X, d, e and g are independently selected within a linker.

Thus, a selected embodiment of the present invention is the following phosphate-binding compound formulas (VII, VIII, IX, X and XI): Formula (VII) (A)(B), no linker; Formula (VIII) (A)-(n)(B), linker is a single covalent bond; Formula (IX) (A)-$[(CH_2)_eC(X)NH(CH_2)_e(NHC(X)(CH_2)_e)_d]$-(B); Formula (X) (A)-$[(C_6R''_4)O)_d(CH_2)_e(C(X)NH(CH_2)_e)_g(NH)_dC(X)NH(C_6R''_4)(CH_2)_e]$-(B) and Formula (XI) (A)-$[NHC(X)(NH)_d(CH_2)_e(NH)_dC(X)(NH)_d(CH_2)_e(NHC(X)(CH_2)_e)_d]$- (B), wherein A is a chemical moiety and B is a metal-chelating moiety.

Any combination of linkers may be used to attach the chemical moiety and the metal-chelating moiety together. In addition, a metal-chelating moiety may have more than one linker that is used to attach either another label, such as an energy transfer pair, or an additional substance such as agarose, a microparticle or a reactive group that functions to attach the linker to the additional substance or to the phosphorylated target molecule. A preferred embodiment includes a metal-chelating moiety attached to a label, with or without a linker, and also attached to an additional substance. The linker may also be substituted to alter the physical properties of the labeling compound, such as binding affinity of the metal-chelating moiety and spectral properties of the dye, or substituted with an amine- or thiol-reactive group.

Another important feature of the linker is to provide an adequate space between the chemical moiety A and the chelating moiety B so as to prevent the chemical moiety from providing a steric hindrance to the binding of the metal ion for the binding domain of the metal-chelating moiety and the binding of the metal ion for the phosphorylated target molecule. It is appreciated that not all chemical moieties will provide a steric hindrance, as a preferred embodiment of the present invention is a metal-chelating moiety that comprises a dye without a linker. However, some labels such as biotin are typically attached to the metal-chelating moiety by a linker. Therefore, the linkers of the present phosphate-binding compounds are important for (1) attaching the chemical moiety A to the metal-chelating moiety, (2) providing an adequate distance between the chemical moiety and the metal-chelating moiety so as not to sterically hinder the affinity of the metal-chelating moiety and a phosphate group on a target molecule and (3) for altering the affinity of the metal-chelating

moiety for the phosphorylated target molecule either by the choice of the atoms of the linker or indirectly by addition of substituents to the linker.

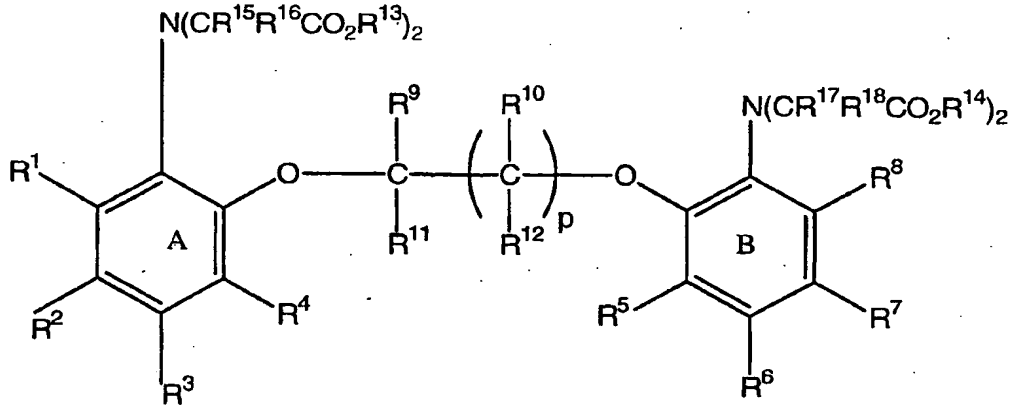
The metal-chelating moieties of the present invention typically contain 1) no linker, 2) a single covalent bond as a linker, 3) a linker of Formula I, 4) a linker of Formula II, 5) or a linker of Formula III. However, it is appreciated that a wide variety of linkers that do not fall within the scope of these formulas are also useful as linkers of the phosphate-binding compounds. These options can be present individually or in any combination, as embodied by the formula (A)m(L)n(B), on the metal-chelating moiety to attach chemical moieties such as labels or reactive groups to form the phosphate-binding compounds of the present invention.

3. Metal-Chelating Moieties

The metal-chelating moieties are moieties that simultaneously bind metal ions and have affinity for exposed phosphate groups on target molecules, wherein a ternary complex is formed between the metal-chelating moiety, the metal ion and the phosphorylated target molecule. Metal ions that have been found to bind phosphate groups include trivalent gallium, iron and aluminum. Metal-chelating moieties that bind these ions, under certain conditions, include BAPTA, IDA, DTPA and phenanthrolines. Thus, the metal-chelating moieties must 1) bind metal ions that have affinity for phosphate groups, 2) not interfere with the binding of the metal ion for the phosphate groups and 3) maintain a stable ternary complex. Metal-chelating moieties that fit these three criteria include BAPTA, IDA, DTPA and phenanthrolines.

BAPTA, as used herein, refers to analogs, including fluorescent and nonfluorescent derivatives, of the metal-chelating moiety (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) and salts thereof including any corresponding compounds disclosed in US Patent Nos. 4,603,209; 4,849,362; 5,049,673; 5,453,517; 5,459,276; 5,516,911; 5,501,980; and 5,773,227. These BAPTA-based metal-chelating moieties are well known to those skilled in the art, primarily as calcium indicators due to their ability to bind divalent calcium ions under physiological conditions, i.e. a pH of about 7 and free calcium ion concentrations near the micromolar and submicromolar range. However, we found that calcium is a totally ineffective metal ion for practice of the methods of the present invention to detect phosphorylated target molecules described in this invention with these indicators.

For the sake of clarity the following structure represents preferred BAPTA metal-chelating moieties of the present invention having Formula IV:



Preferably ring A and ring B are linked by a hydrocarbon bridge between two oxygen atoms in which p is 0 or 2 and the ring substituents (R^1–R^8) are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alkoxy, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano, thioether, sulfinyl and linker (L). Alternatively, two adjacent ring substituents in combination constitute a cyclic substituent that is cycloalkyl, cycloheteroalkyl, aryl, fused aryl, heteroaryl or fused heteroaryl. Preferably, the BAPTA metal-chelating moieties have at least two substituents that are not hydrogen, a most preferred BAPTA metal-chelating moiety is substituted by a fluorine atom as one of the substituents, most preferably substituted at the R^6 or R^3 position (e.g., Compounds 1, 2, 5, 7, 8 and 12). Typically the linker attaching the chemical moiety to the BAPTA is at the R^3 or R^6 position. Equally preferred are BAPTA metal-chelating moieties that comprise a carbonyl group as a substituent, preferably at the R^7 position, e.g., Compounds 9 and 12. Without being bound by a particular theory, it appears that an electron withdrawing group such as fluorine or carbonyl substituted at the R^3, R^4, R^6 or R^7 position results in BAPTA chelating moieties that are particularly advantageous for chelating trivalent gallium ions that then also allows for the simultaneous interaction of the chelated gallium ion with an exposed phosphate group on the phosphorylated target molecules, resulting in a stable ternary complex.

The bridge substituents R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen, lower alkyl, or adjacent substituents R^9 and R^{10}, taken in combination, constitute a 5-membered or 6-membered alicyclic or heterocyclic ring. R^{15},

R^{16}, R^{17} and R^{18} are independently H or lower alkyl; preferably R^{15}, R^{16}, R^{17} and R^{18} are all hydrogen. R^{13} and R^{14} are independently hydrogen or a salt.

It is understood that the chemical moieties of the present invention are attached to the BAPTA metal-chelating moiety by a linker at any or R^1–R^{12} or dye label comprises one of the aromatic rings of the metal-chelating moieties wherein no linker is present. Therefore, two adjacent substituents of R^1–R^{12}, when taken in combination with each other, and with the aromatic ring to which they are bound, comprise a fluorophore or chromophore label. However, a phosphate-binding compound could have more that one linker, such that a dye label is attached with no linker and four other linkers are present on the metal chelating compound to attach other labels or reactive groups. In one aspect of the invention, two adjacent ring substituents (R^1–R^4 or R^5–R^8) taken in combination form the dye label that is a fused benzofuran or heteroaryl- or carboxyheteroaryl-substituted benzofuran fluorophore. Where the dye label is fused to the compound of the invention, it is preferably fused between R^2 and R^3, or between R^6 and R^7.

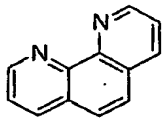
Xanthene derivative dyes are particularly useful dyes of the present invention wherein, either or both of the benzene rings of the BAPTA or substituted BAPTA metal-binding compound is bonded to a xanthene ring through a single chemical bond, as in the common Ca^{2+} indicators fluo-3, fluo-4 and rhod-2 (US Patent No. 5,049,673, *supra*) or through the intermediacy of a phenyl or substituted phenyl spacer as in the Oregon Green® BAPTA indicators (US Patent No. 6,162,931, *supra*). The xanthene rings are typicallly bonded to the BAPTA at positions *para* to the nitrogen functions of the BAPTA. Particularly preferred are xanthene-containing BAPTA derivatives whose fluorophore is a rhodamine or a halogenated fluorescein. Particularly preferred are fluorescent BAPTA derivatives in which the 5-position of the BAPTA chelator is substituted by F, including rhod-5F and fluo-5F.

DTPA, as used herein, refers to diethylenetriamine pentaacetic acid chelating moieties and derivatives thereof, including any corresponding compounds disclosed in US Patent Nos. 4,978,763 and 4,647,447. DTPA metal-chelating moieties are represented by Formula V comprising $(CH_2CO_2R^{13})_2N[(CH_2)_sN(CH_2CO_2R^{13})]_T(CH_2)_sN(CH_2CO_2R^{13})_2$, wherein a linker is attached to a methine carbon or nitrogen atom, Z is 1 or 2, S is 1 to 5, T is 0–4 and R^{13} is independently a hydrogen or a salt.

IDA, as used herein, refers to iminodiacetic acid compounds and derivatives thereof and is represented by Formula VI comprising -(L)-$N(CH_2CO_2R^{13})_2$ wherein R^{13} is independently a hydrogen or a salt and provided that said linker is not a single covalent bond. The IDA

metal-chelating moieties must be attached by a linker to a chemical moiety wherein the linker comprises at least one nonhydrogen atom. Without wishing to be bound by a theory, it appears that the linker increases the stability of the ternary complex and possibly tunes the affinity of the metal-chelating moiety for a metal ion of the present invention.

In addition to the above mentioned specific metal chelating moieties we have also found that phenanthroline based chelators also form ternary complex with metal ions and phosphate target molecules in a moderately acidic environment. Phenathroline, as used herein, refers to 1,10-phenanthroline compounds and derivatives thereof and is represented by the structure



Any of the aromatic carbon atoms may be substituted with substituents well known to one skilled in the art, including those substituents disclosed in US Patent 6,316,267, *supra*. Alternatively, a linker can be attached to any of the aromatic carbon atoms to covalently attach a chemical moiety A to the phenanthroline moiety to form the phosphate-binding compounds of the present invention.

B. Phosphate-binding compounds

The synthetic strategy of phosphate-binding compounds that provide optimal signals after formation of a ternary complex involves selection of appropriate chemical linkages between the chemical moieties A and the metal-chelating moiety, and also selection of appropriate substituents on the metal-chelating moiety. These selections are made such that the resulting phosphate-binding compound retains optimal simultaneous binding affinity for both the metal and of the metal for the phosphorylated target molecules and sufficient solubility to promote a persistent ternary complex. Improper selections result in phosphate-binding compounds that do not have sufficient binding affinity and do not produce a persistent ternary complex. Improper selections also result in excessive non-selective binding of the phosphate-binding compound to analytes other than the phosphorylated target compounds, resulting in a high background and thus a low signal-to-noise ratio. Compounds that are suitable for practice of the invention are best screened by the method in Example 1D.

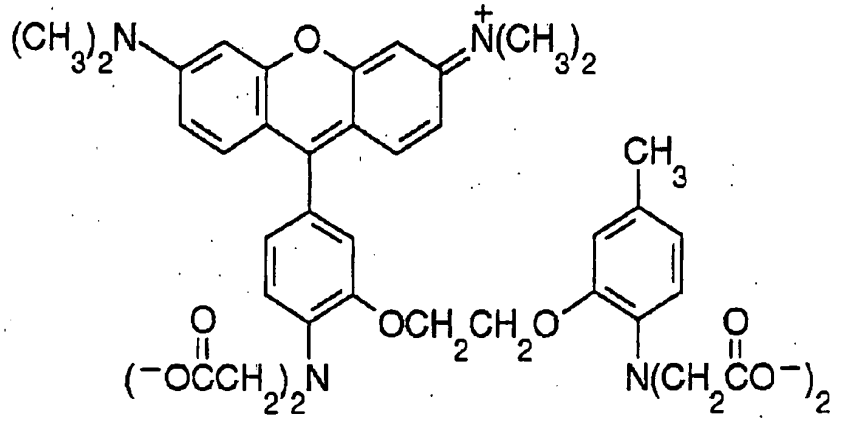
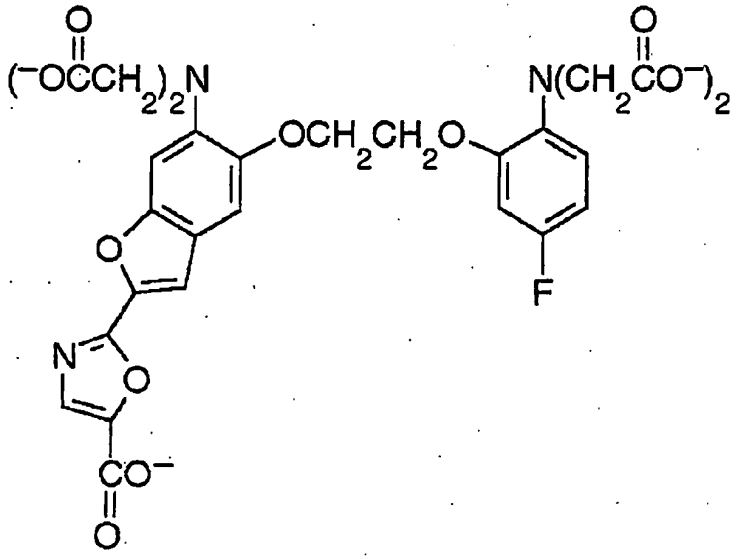
We have discovered that BAPTA derivatives are particularly suitable for practice of the various aspects of the invention. The novel phosphate-binding compounds of the present invention whose synthesis and use is illustrated in examples include BAPTA chelating

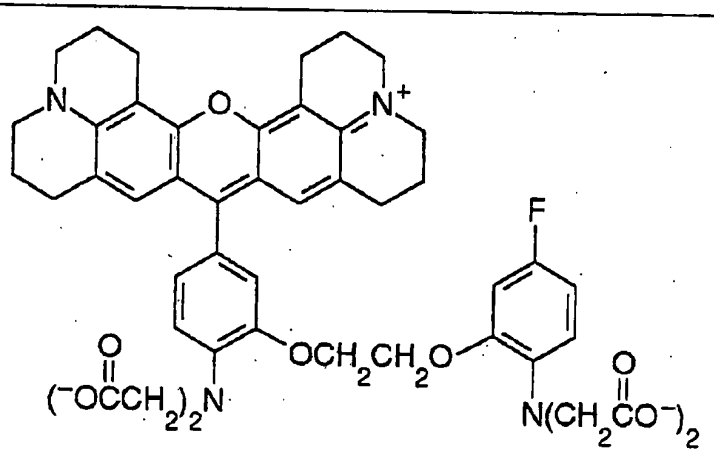
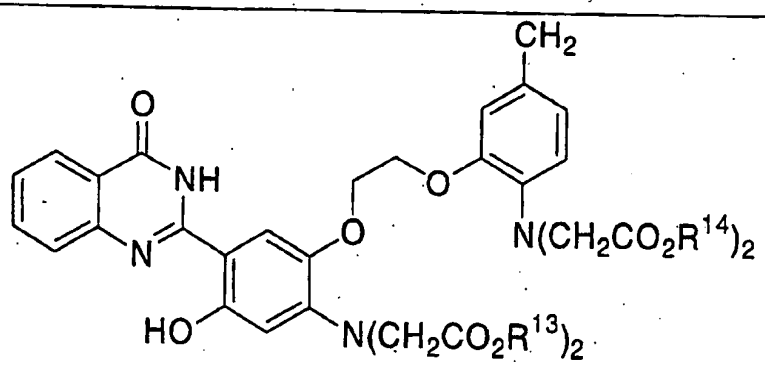
moieties with a quinazolinone fluorescent dye (Compounds 6, 7 and 23), BAPTA chelating moieties with a borapolyazaindacene fluorescent dye (Compounds 8 and 24), BAPTA chelating moieties with a biotin label, wherein the biotin is attached by a linker (Compounds 9, 12, 15 and 18), BAPTA chelating moieties with a benzothiazole label (Compound 17), BAPTA chelating moieties with agarose covalently attached (Compounds 13 and 14), BAPTA compounds comprising an aniline attached by a linker to the BAPTA compound (Compound 10). Novel compounds also include borapolyazaindacene fluorophore labels attached by a linker to DTPA chelating moieties (Compounds 20, 21 and 22). These novel phosphate-binding compounds find use in the detection and isolation of phosphorylated target molecules. Synthesis of these compounds is exemplified in Examples 28–43.

The phosphate-binding compounds of the present invention exhibit sufficient noncovalent binding affinity for the gallium (III)-phosphorylated target molecule complex to allow for rinsing away of excess reagents from the persistent ternary complex. Additionally, it was found that certain phosphate-binding compounds provided optimal signal after formation of the ternary complex and are thus more environmentally sensitive. This high signal appears to be a function of well-tuned hydrophobicity of the phosphate-binding compound–gallium (III)–phosphorylated target molecule complex. Therefore, when a detectable response is desirable, e.g., labeling phosphorylated target molecules in solution, and where the detectable response is a fluorescence response, it is typically a change in fluorescence, such as a change in the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. Preferably, the detectable optical response upon binding the gallium ion and the phosphorylated target molecule to the chelator is a change in fluorescence intensity that is greater than approximately 2-fold.

However, for applications wherein the phosphorylated target molecule or phosphate-binding compound is immobilized — resulting in an immobilized ternary structure — an increase in detectable fluorescence response due to the chelation of the metal-chelating moiety and subsequent ternary complex formation is not necessary. This is due to the stable ternary complex, which allows for washing and removal of unbound phosphate-binding compounds wherein the fluorescence response from the phosphate-binding compound is sufficient to visualize the phosphorylated target molecule. Therefore, a preferred embodiment in this situation is a phosphate-binding compound that undergoes little or no change in fluorescence when bound to a metal ion of the present invention and a phosphorylated target molecule.

	Compou	number	Compou		Compou

Compound 3		The fluorophore is a xanthene (rhodamine) derivative and the metal-chelating moiety is a BAPTA compound (Formula IV). The linker is a single covalent bond. The counterion is K^+.
Compound 4		The fluorophore is a benzofuran that shares an aromatic ring with the metal-chelating moiety (Formula IV) and comprises a substituted heteroaryl moiety. The metal-chelating moiety, BAPTA, is fluorinated at the R^6 position. The phosphate-binding compound does not comprise a linker. The counterion is K^+.

Compound 5		The fluorophore is a xanthene (rhodamine) derivative and the metal-chelating moiety is a BAPTA compound (Formula IV) that is fluorinated at the R^6 position. The linker is a single covalent bond. The counterion is K^+.
Compound 6		The fluorophore is a quinazolinone with an adjacent hydroxyl group on the metal-chelating moiety (BAPTA, Formula IV). R^{13} and R^{14} are independently hydrogen or a salt and the linker is a single covalent bond.

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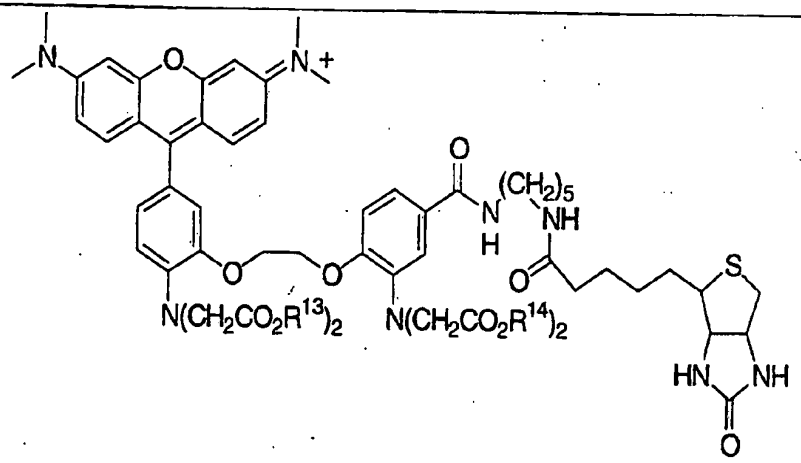
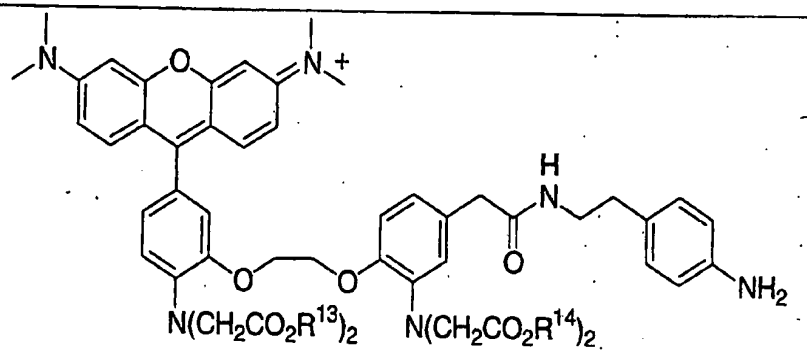
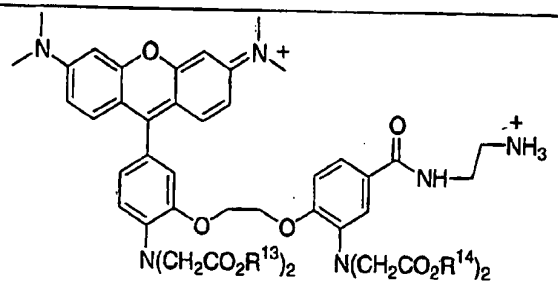
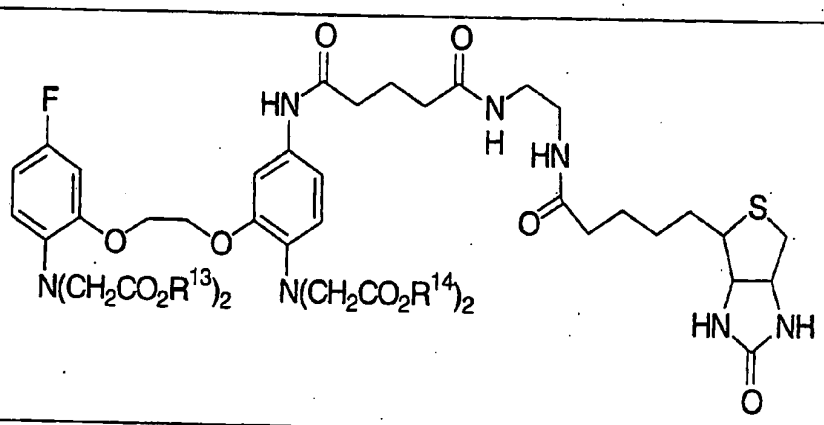
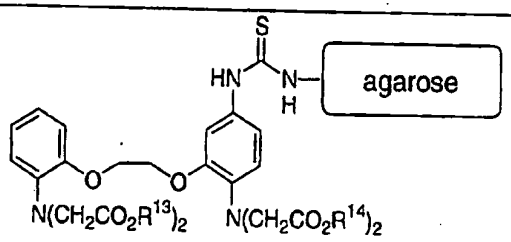
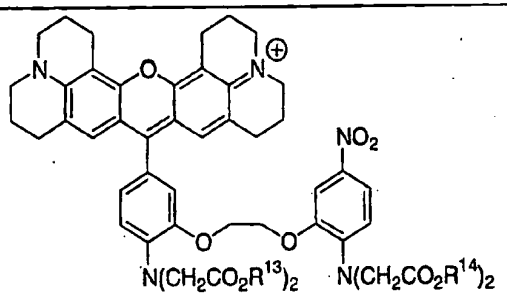
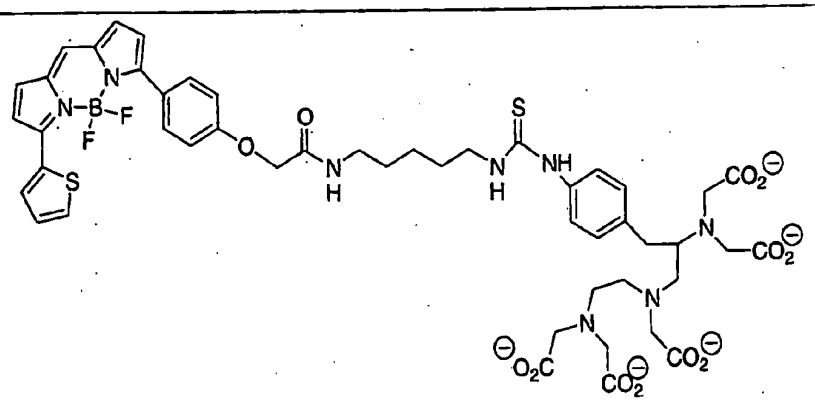
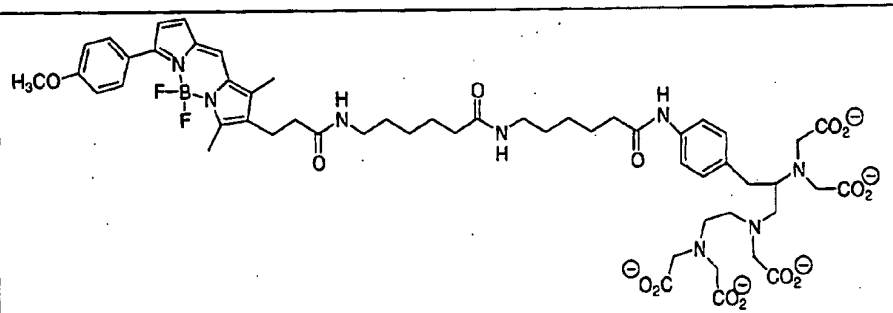
Compou

Compou

Compound 9		The fluorophore is a xanthene (rhodamine) derivative and the metal-chelating moiety is a BAPTA compound (Formula IV), wherein R^{13} and R^{14} are independently hydrogen or a salt. The linker attaching the fluorophore to the BAPTA compound is a single covalent bond. A second linker at R^7 (Formula I) covalently attaches a biotin label to the phosphate-binding compound.
Compound 10		The fluorophore is a xanthene (rhodamine) derivative that is attached by a single covalent bond linker to the metal-chelating moiety (BAPTA, Formula IV), wherein R^{13} and R^{14} are independently hydrogen or a salt. A second linker (Formula I) at R^7 attaches an aniline moiety.

Compound 11		The fluorophore is a xanthene (rhodamine) derivative that is attached to the metal-chelating moiety (BAPTA, Formula IV) by a single covalent bond, wherein R^{13} and R^{14} are independently hydrogen or a salt. A second linker (Formula I) at R^7 attaches an amine group.
Compound 12		The label is a biotin that is attached to the metal-chelating moiety (BAPTA, Formula IV) by a linker (Formula III). The metal-chelating moiety is fluorinated at the R^3 position and R^{13} and R^{14} are independently hydrogen or a salt.
Compound 13		The metal-chelating moiety (BAPTA, Formula IV) is attached to agarose by a linker, wherein R^{13} and R^{14} are independently hydrogen or a salt.

PATENT

Compound 16		The metal-chelating moiety (BAPTA, formula IV) is attached by a single covalent bond to a xanthene (rhodamine) derivative. R^{13} and R^{14} are independently hydrogen or a salt.
Compound 20		The dye is a borapolyazaindacene that is attached to the metal-chelating moiety (DTPA, Formula V) by a linker (Formula II). The dye is substituted by a thienyl group.
Compound 21		The dye is a borapolyazaindacene that is attached to a metal-chelating moiety (DTPA, Formula V) by a linker (Formula II).

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The metal ion—containing salt preferably contains trivalent gallium ions, such as is prepared from gallium chloride, but can be any gallium salt known to those skilled in the art. Alternatively iron and aluminum ions also find use in the binding solution of the present invention. Gallium salts that can be used with the present invention include, without limit, acetylacetonate, arsenide, bromide, chloride, fluoride, iodide, nitrate, nitride, perchlorate, sulfate and sulfide. The gallium salt is typically present in the binding solution at a concentration of about 10 nM to about 1 mM; preferably the concentration of the gallium salt is about 0.5 μM to 10 μM. However for precipitation purposes, the gallium salt is preferably present at a slightly higher concentration of about 0.1 mM to about 0.5mM.

Analysis of the stability and specificity of the phosphate-binding compounds for gallium ions and the gallium ions for the phosphorylated target molecules was evaluated as a function of pH (Example 1). Based on these results, it was determined that a preferred bidning solution comprises an acid to provide a moderately acidic environment for the binding reaction. In fact, an important and unexpected aspect of the present invention is that metal-chelating groups bind trivalent cations such as gallium in a moderatly acidic environment, resulting in a titration of fluorescent signal with an increase in pH level approaching neutral pH. An acidic environment is defined as a solution having a pH less than 6.9. Typical suitable acidic components include without limitation acetic acid, trichloroacetic acid, trifluoroacetic acid, perchloric acid, or sulfuric acid. The acidic component is typically present at a concentration of 1%–20% and is buffered to the appropriate pH by a base. The pH of the binding solution is preferably about pH 3–6 and most preferred is about pH 4.0. Acetic acid is a preferred acid for use at or near pH 4. The optimal pH for each compound used may vary slightly depending on the compound used, for Compound 2, pH 4.0 is preferred.

The pH of the binding solution is optionally modified by the inclusion of a buffering agent in addition to the acidic component. In particular, we have shown that the presence of a buffering agent unexpectedly improves binding of phosphorylated targetmolecules immobilized in electrophoresis gels, provided that an alcohol is also included in the formulations. Any buffering agent that maintains an acidic environment and is compatible with the phosphorylated target molecules in the sample is suitable for inclusion in the binding solution.

Useful buffering agents include salts of formate, acetate, 2-(*N*-morpholino)ethanesulfonic acid, imidazole, *N*-(2-hydroxyethyl)piperazinylethanesulfonic acid, *tris*-(hydroxymethyl)aminomethane acetate, or *tris*(hydroxymethyl)aminomethane, hydrochloride,

immobilized on a membrane comprises the following steps:

solution the gels or membranes are typically washed with a mixture that preferably comprise

MDPF and CBQCA could also be used for detection on membranes. Because SDS is

information can be readily retrieved in tabular form with differential expression data calculated.

Alternatively, single-dimension polyacrylamide and corresponding blots can be simultaneously or subsequently stained for total proteins or glycoproteins using staining techniques and dyes described above. A particular advantage for counterstaining a gel or blot that has been labeled using methods of the present invention is the ability to distinguish between nonspecific labeling and labeling of phosphorylated target molecules with a low number of phosphate groups. This is important for accurately identifying phosphorylated target molecules that have undergone a small change in the degree of phosphorylation. Counterstaining a blot or gel with a total protein stain such as SYPRO® Ruby permits a ratiometric analysis of the fluorescent signal generated from the dyes of the present invention compared to the fluorescent signal generated from a total protein stain (*see*, Figure 11 and Example 22). This ratiometric analysis also permits the stoichiometry determination of the phosphorylated target molecule relating to the overall phosphorylation state of the molecule as well as the addition or subtraction of phosphate groups.

Another particular advantage for staining phosphorylated proteins or peptides separated in polyacrylamide gels is for the analysis of proteins of interest by combining spot detection with the compounds of this invention with mass spectrometry techniques for further analysis. For example, because phosphoproteins may co-migrate in a gel, further analysis may be essential or desired to specifically identify and analyze the phosphoprotein of interest. This further analysis can be achieved by measurement of a set of peptide masses derived from a protein, i.e., by peptide mapping with mass spectrometry (MS), or by obtaining amino acid sequence information from individual peptides, i.e., protein sequencing by MS/MS or by Edman degradation. Thus, a protein band or spot, once identified using the compositions and methods of the present invention, may be excised from the gel, rinsed, optionally reduced and *S*-alkylated, and then digested *in situ* in the gel with a sequence-specific protease, e.g., trypsin, using standard protocols. *See* Shevchenko *et al.*, "Mass Spectrometric Sequencing of Proteins from Silver Stained Polyacrylamide Gels," *Anal. Chem. 68*:850-58 (1996). The peptide mixture thus generated may be extracted from the gel and analyzed by MS, using standard protocols. Peptide mapping by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is often most sensitive. Methods for the in-gel digestion of proteins are described in Jensen *et al.*, "Mass Spectrometric Identification and Microcharacterization of Proteins From Electrophoretic Gels: Strategies and Applications," *PROTEINS: Structure, Function, and Genetics Suppl. 2*:74-89 (1998).

DNA-binding proteins are key to the regulation and control of gene expression, replication and recombination. The electrophoretic mobility shift assay (or gel shift assay) is considered an essential tool in modern molecular biology for the study of protein–nucleic acid interactions. Nucleic acids could be detected with SYBR® Green II dye, while phosphoproteins are subsequently detected by methods described in this invention. All fluorescence staining steps would be performed after the entire gel-shift experiment is completed, so there is no need to pre-label either the DNA or the protein and no possibility of the fluorescent reagents interfering with the protein-nucleic acid interactions. A third total protein stain might be employed as well, such as SYPRO® Ruby dye. In this way the influence of protein phosphorylation on DNA-binding may be measured. The ability to independently quantify each molecular species allows more rigorous data analysis methods to be applied, especially with respect to the mass of phosphoprotein bound per nucleic acid.

The present invention is also contemplated to be used in a wide range of microarray formats, including but not limited to the methods and arrays disclosed in US Patent Application 2002/0076727; US Patent Application 2002/0106785; US Patent Application 2002/0055186; WO 99/39210; WO 00/63701; WO 02/25288; WO 01/18545, WO 00/04380 and US patent Nos. 6,403,368; 6,475,809; 6,365,418; 6,409,921; 5,595,915; 6,461,807; 6,399,299. Phosphorylated target molecules immobilized on an array such as a HydroGel-coated slide including those disclosed in US Patent Nos. 6,372,813; 6,391,937; 6,387,631; 6,413,722 and those manufactured by Perkin Elmer; can also be detected using the methods and compositions of the present invention (Examples 18 and 19). Alternatively, phosphate-binding compounds can be immobilized on these arrays.

The methods of the present invention for detecting phosphorylated target molecules on an array comprise the steps of:

i) immobilizing said sample on an array;

ii) contacting said array of step i) with a binding solution,

iii) incubating said array of step ii) and said binding solution for sufficient time to allow said compound to associate indirectly with said phosphorylated target molecule; and,

iv) illuminating said compound with a suitable light source whereby said phosphorylated target molecule is detected.

The sample is immobilized on the array using techniques well known to one skilled in the art, including but not limited to, using a piezo array printer or other array printer technology, immobilizing a phosphorylated target molecule binding molecule such as an antibody and then added the sample to non-covalenlty bind the phosphorylated target molecules to the array. Typically the array comprises molecules that covalenly attach the sample, or a protein that selectively binds the sample, such as an amine reactive group.

The array is incubated with a binding solution for sufficient time to form a ternary complex between a phosphate-binding compound, the metal ion (typically gallium) and phosphorylated target molecule. Alternatively, the array may comprise phosphate-binding compounds complexed with the metal ions immobilized on the surface of the array, wherein a sample is incubated with the array and detection of phosphorylated target molecules occurs when the target molecules bind the metal ion/phosphate-binding complex and are typically illuminated, unbound sample is washed away. In this way, an assay to detect phosphatases or kinases is performed with an appropriate peptide or protein substrate and the resulting phosphorylated or dephosphorylated peptides or proteins are spotted or synthesized on the array, wherein phosphate groups on the peptides bind the phosphate-binding compounds/metal-ion complex on the array. Alternatively, a kinase and/or phosphatase substrate is spotted or synthesized on the array and then the enzyme, kinase (and ATP) or phosphatase, is added to the array. After removing the enzyme, the array is then contacted with the binding solution. In this way, the array is used to detect and/or isolate phosphorylated target molecules and to identify the enzymes responsible for adding and/or removing phosphate groups from target molecules and their efficiency in doing so.

Typically phosphatase and kinase peptide substrates are immobilized on an array by spotting or synthesis using standard protocols, the phosphates or kinase enzymes, either comprise an unknown sample or are isolated enzymes, are added and subsequent presence of phosphate groups is detected using a binding solution of the present invention. Thus, the methods and binding solution of the present invention are useful, for example, with arrays of protein substrates for various protein kinases (e.g., myosin light chain, MARCKS, myelin basic protein, casein, src-supressed C kinase substrate, insulin Receptor Substrate 1, Nuclear factor 90, Rap1, transcription factor stat5a). A sample comprising phosphatase or kinase enzymes is incubated with the array comprising enzyme substrate; following incubation under appropriate conditions and with appropriate reaction additives for the enzymes the phosphorylated products can be detected with a binding solution of the present invention. If the detectable label is a fluorophore, for example, the coordinates of the

fluorescent signals would provide a read-out of the kinases present in the fluid and their activity against the various enzyme substrates (peptides or proteins) on the array. Detection of phosphorylated target molecules with an array offers many possibilities and the above description is not meant to limit how the present invention can be used in combination with array technology.

Current commercial kinase assays are often time-consuming and require many steps such as electrophoresis, centrifugation, ELISA or immunoprecipitation. The present invention provides methods for the rapid, sensitive, and non-radioactive detection of a variety of selected kinases and phosphatases and provides, in addition, methods that are well suited for high-throughput screening. The kinase and phosphatase assays of the present invention will also permit the screening of inhibitors and activators of, for example, tyrosine kinases and, in addition, will also permit the monitoring and the purification of kinase and phosphatase enzymes. Moreover, detection of the enzyme substrate on the array makes the methods of the invention far more sensitive than any known solution-based assays for kinases and phosphatases.

As described above, a kinase substrate is covalently or non-covalently attached to a surface, solid or semisolid matrix including a microwell plate, polymeric beads or an array such as a HydroGel array slide and the assay is performed in a non-continuous heterogeneous manner. The kinase substrate comprises a kinase consensus phosphorylation site, preferably a peptide or a random polymer (poly(Glu:Tyr), poly(Glu:Ala:Tyr). Optionally the kinase substrate comprises a fluorophore. A sample suspected of containing a kinase is combined with the kinase substrate, along with ATP, wherein an active kinase enzyme will add phosphates to the kinase substrate. The addition of phosphate groups is measured after removal of the kinase solution and adequate washing wherein a binding solution, as described above, is added to the kinase substrate. Typically the phosphate-binding compound comprises a fluorophore and the kinase activity is measured by illuminating the fluorophore. Alternatively, the phosphate-binding compound comprises an enzyme such as peroxidase, wherein the kinase activity would be measured after addition of the appropriate enzyme substrate and detection with a fluorometers or an instrument to measure color or chemiluminescence. In addition, using an inhibitor of the selected kinase or phosphatase in the assay, for example, by using sodium orthovanadate may enhance the specificity of the kinase. Furthermore, the assay methods of this invention can be used to screen for inhibitors or activators of kinases and/or phosphatases. Alternatively, the assay is easily adaptable to measure phosphatase activity wherein phosphatase substrate, phosphorylated

subsequently bound with the binding solution of the present invention, resulting in

Unexpectedly, phosphorylated target molecules, typically peptides, can also be isolated from a complex solution by taking advantage of the insoluble nature of the ternary complex when higher concentrations of the more hydrophobic phosphate-binding compounds, such as Compound 5, are used in a moderately acidic environment with equimolar metal ion concentrations.

Methods of the present invention for isolating phosphorylated target molecules from solution comprise the following steps:

i) contacting the sample with a binding solution of the present invention;

ii) incubating the sample of step i) and the binding solution for sufficient time to allow said compound to associate with the phosphorylated target molecule to form a ternary complex; and,

iii) separating said complex from said sample, whereby said phosphorylated target molecules are isolated.

Hydrophobic phosphate-binding compounds of the invention when present in a binding solution at a concentration up to a hundred times higher than a binding solution for detection purposes will form insoluble aggregates when the ternary complex forms. This property of the certain hydrophobic phosphate-binding compounds was taken advantage of to develop a method for isolation of phosphopeptides. Thus, when a binding solution comprising certain hydrophobic phosphate-binding compounds is incubated with a sample in a way to facilitate formation of the ternary complex, the complex can be precipitated out of solution by centrifugation (Example 13). Therefore, typically the binding mixture and sample solution is vortexed, or mixed in a manner well known to those skilled in the art, to simultaneously facilitate binding (formation of aggregates) and prevent precipitation of the ternary complexes. Following formation of the ternary complex, the solution is treated in such a way as to isolate the precipitated complexes, wherein a preferred method is centrifugation. The resulting pellet comprises phosphorylated target molecules that can be further analyzed, by methods such as MS. This method takes advantage of the affinity "pull-down" of phosphopeptides or phosphoproteins from a complex solution (e.g., a cell extract protein digest), whereby at an acidic pH phosphate-binding compounds can complex with metal (typically gallium) ions and the phosphopeptides or phosphoproteins to form a precipitate. In addition, for the methods used to precipitate phosphorylated target molecules from solution, aluminum ions and ferric chloride comprising iron ions can be also used for the formation of the ternary complex.

In addition to isolation of phosphorylated target molecules from a complex sample in solution, the present invention also contemplates the isolation of target molecules by capturing the phosphorylated target molecules using immobilized phosphate-binding compounds (Example 15, 25 and 26). This can be done in a number of ways and the method is exemplified using an affinity column, ferrofluid beads and membranes; however, the methods illustrated are not intended to be a limitation of the method.

The methods of the present invention for isolating phosphorylated target molecules from solution using immobilized phosphate-binding compounds comprise the following steps:

i) charging a matrix comprising an immobilized phosphate-binding compound, wherein a metal-chelating moiety comprises Formula IV, with a salt comprising metal ions;

ii) equilibrating the matrix with a moderately acidic binding buffer,

iii) adding the sample to the matrix, wherein the phosphorylated target molecules are bound to the matrix of step ii); and

iv) eluting the phosphorylated target molecules from the matrix, whereby said phosphorylated target molecules are isolated.

The matrix can be any matrix known to one skilled in the art, including polymeric membranes, polymeric particles such as agarose, latex, magnetic or Sepharose beads, and glass, such as slides, beads or optical fibers. The beads can be present in slurry or as a packed column through which the sample passes and the membranes capture the phosphorylated target molecules. An example of such a column is an affinity matrix comprising phosphate-binding compounds bound to agarose (for instance, Compounds 13 and 14) or a resin (immobilized affinity column (IMAC)). Other compounds that find use in this method include, among others, Compounds 15, 20, 21 and 22)

Unlike the precipitation method, where an affinity column or organic extraction buffer can be used to remove phosphate-binding compounds from the isolated phosphorylated target molecules, the matrix in this method can optionally comprise just the metal-chelating moiety component of the phosphate-binding compound, which is subsequently bound with metal (preferably gallium) ions following the addition of the metal salt. However, a phosphate-binding compound represented by formula (A)m(L)n(B) can form the matrix wherein A is a reactive group that is used to attach B by way of L to the matrix material. Thus, the matrix is

charged with the metal ion, prior to addition of the sample. The matrix is then equilibrated with a moderately acidic binding buffer; alternatively, the metal ion and acidic binding buffer would be present in one solution. The acidic binding buffer typically uses the same components as the binding solution. A sample in an acidic binding buffer is then added to the mixture, where phosphorylated target molecules will bind the metal ions complexed to the metal-chelating moiety. Isolation of the phosphorylated target molecules is accomplished by an addition of a solution, which dissociates the ternary complex of the phosphate-binding compound (metal-chelating moiety), metal ion and phosphorylated target molecules. Preferably, the elution solution comprises a base and a basic pH-buffering agent. Useful bases include, without limitation, barium hydroxide, sodium hydroxide and ammonia hydroxide. Alternatively, basic amine solutions are also useful elution agents. Any base that is compatible with the sample and metal ion phosphorylated target molecule complex that dissociates the complex is preferred. In addition, organic solvents such as acetonitrile is useful in eluting phosphorylated target molecules from the phosphate-binding compound matrix, and may be preferable, depending on the subsequent analysis of the phosphorylated target molecules, such as with MS.

As many phosphorylated target molecules often exist only in low abundance, the isolation methods of the present invention are especially useful for the purification and enrichment of such phosphorylated target molecules. These methods are useful for purifying phosphorylated peptides from crude peptide mixtures, which is advantageous for methods that subsequently analyze the peptides by MALDI, MS or nanoelectrospray tandem mass spectrometry (MS/MS). It is contemplated that a wide variety of methods can be used to prepare samples purified and/or enriched by the affinity matrix or separated from a complex solution. For example, dried separated phosphopeptides can be resuspended in water for LC-MS analysis.

The IMAC of the present invention is also readily adaptable to microfluidics applications, such as the CD technology developed by Gyros AB (Uppsula, Sweden), wherein high-throughput screening of samples for proteomic analysis, such as peptide mapping with MALDI-TOF, can be accomplished. Briefly, the Gyros AB technology comprises a CD microlaboratory with hundreds of microstructures (columns), wherein samples are run through the columns based on centrifugation speeds and the eluted sample is analyzed on the CD, permitting the entire process from a protein digest to MS analysis to be conducted on the CD. The columns can be packed with particles that comprise BAPTA compounds (Compounds 13 or 14); samples can be then run through the columns and either analyzed

with well-known methods of magnetic bead separation.

Thus, a wide variety of materials and methods are provided for the separation, purification and enrichment of phosphorylated target molecules, including the novel use of an immobilized affinity matrix.

The present invention provides compounds and methods for the differential isolation and identification of phosphorylated serine, threonine or tyrosine amino acids. The materials and methods described above for the labeling and isolation of phosphorylated target molecules, absent mass spectrometry or other similar techniques, are generally used for detecting protein phosphorylation, but do not give information on the specific location of the phosphate on the protein or polypeptide. The present invention contemplates further analyzing isolated phosphorylated proteins or peptides obtained by immobilized affinity matrix or precipitation methods described above to differentially identify phosphorylated peptides. Isolated phosphorylated proteins are subjected to proteolytic digestion, followed by acid hydrolysis or alkaline hydrolysis and analyzed.

A base such as barium hydroxide or sodium hydroxide catalyzes the dephosphorylation of the peptides, forming activated dehydroalanine derivatives, which are vulnerable to attack by amine or thiol containing compounds, resulting in the formation of stable derivatives of the original phosphopeptide. These derivatives are more hydrophobic and are therefore more amenable to identification by HPLC, mass spectrometry, or by Edman sequencing. Under the conditions used, phosphoserine residues undergo elimination and addition, phosphothreonine residues undergo elimination but not addition and phosphotyrosine residues are unaltered by the treatment. Thus, differential identification can be accomplished based on this knowledge. In Edman degradation, during the acid or base delivery the phosphate is β-eliminated and the resulting dehydro-amino acids rapidly form a dithiothreitol (DTT) adduct. *See* Meyer *et al.*, *FASEB J.* 7:776 (1993). In contrast, *O*-Hex-*N*-Ac on serine and threonine is stable in Edman degradation. *See* Gooley & Williams, *Nature 358*:557 (1997). Thus, the present invention may be used to differentiate between serine or threonine phosphorylation and glycosylation.

Edman degradation is thus an effective method for quantitating serine and threonine, following β-elimination and derivatization. See Yan *et al.*, "Protein Phosphorylation: Technologies for the Identification of Phosphoamino Acids", *J. Chromatogr.* 808:23-41 (1998)). These modified products also survive acid hydrolysis, and can be quantitated by reversed-phase HPLC analysis. *See, e.g.*, Meyer *et al.*; *J. Chromatogr. 397*:113 (1987) and

Holmes, *FEBS Lett. 215*:21 (1987). Using a similar approach, characterization by capillary zone electrophoresis and laser-induced fluorescence has also been used to quantitate the phosphoserine content of peptides and proteins. *See*, Fadden & Haystead, *Anal. Biochem.* 225:81 (1995).

Nanoelectrospray MS/MS is used for phosphopeptide sequencing for exact determination of phosphorylation sites. *See* Stensballe *et al.*, "Characterization of Phosphoproteins From Electrophoretic Gels by Nanoscale Fe(III) Affinity Chromatography With Off-Line Mass Spectrometry Analysis," *Proteomics 1*:207-222 (2001). In-gel digestions can be achieved as described in Shevchenko *et al.*, *Anal. Chem. 68*:850-58 (1996) and Jensen *et al.*, *Meth. Mol. Biol. 112*:513-30 (1998). The present invention also contemplates that the materials and methods can be used with mass spectrometry techniques yet to become available that achieve the same results.

The sequence of phosphopeptides and the identification of the site(s) of phosphorylation can also be determined by a combination of tandem mass spectrometry and computer-assisted database search programs, such as SEQUEST (Trademark, University of Washington, Seattle WA) (McCormack *et al.*, "Direct Analysis and Identification of Proteins in Mixtures by LC/MS/1\4S and Database Searching at the Low-Femtomole Level," *Anal. Chem. 69*:767-776 (1996); Eng *et al.*, "An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database," *J. Amer. Soc. Mass. Spectrom. 5*:976-989 (1994); U.S. Patent No. 5,538,897. While a variety of MS methods are available and may be used in these methods, MALDI/MS and Electrospray Ionization MS (ESI/MS) methods are typically used.

2. Sample preparation

The sample is defined to include any material that may contain phosphorylated target molecules, substrates that interact with kinases and phosphatases, substances that interact with kinase and phosphatase substrates and any substance that binds phosphorylated target molecules. Typically the sample is biological in origin and comprises tissue, a cell or a population of cells, cell extracts, cell homogenates, purified or reconstituted proteins, recombinant proteins, fusion proteins, bodily and other biological fluids, viruses or viral particles, prions, subcellular components, or synthesized peptides or proteins. Possible sources of cellular material used to prepare the sample of the invention include, without limitation, plants, animals, fungi, bacteria, archae, or cell lines derived from such organisms.

The sample can be a biological fluid such as whole blood, plasma, serum, nasal secretions, sputum, saliva, urine, sweat, transdermal exudates, cerebrospinal fluid, or the like. Alternatively, the sample may be whole organs, tissue or cells from an animal. Examples of sources of such samples include muscle, eye, skin, gonads, lymph nodes, heart, brain, lung, liver, kidney, spleen, solid tumors, macrophages, mesothelium, and the like.

Prior to combination with the binding solution of the present invention, the sample is prepared in a way that makes the phosphorylated target molecules or enzyme substrates in the sample accessible to the phosphate-binding compounds. Alternatively, the sample may comprise enzymes or binding proteins that interact with phosphorylated target molecules. Typically, the samples used in the invention comprise tissue, cells, cell extracts, cell homogenates, purified or reconstituted proteins, peptides, recombinant proteins, biological fluids, lipids, amino acids, nucleic acids and carbohydrates or synthesized proteins. However, the desired target (target molecule comprising exposed phosphate groups) may require purification or separation prior to addition of the binding solution due to the presence of other discrete biological components. The desired phosphorylated target molecules and other discrete biological components can be optionally separated from each other or from other components in the sample by mobility (e.g., electrophoretic gel or capillary) by size (e.g., centrifugation, pelleting or density gradient), or by binding affinity (e.g., to a filter membrane or affinity resin) in the course of the present methods. For example, when the sample is to be separated on an SDS-polyacrylamide gel, the sample is first equilibrated in an appropriate buffer, such as an SDS-sample buffer containing Tris, glycerol, DTT, SDS, and bromophenol blue. For certain aspects of the invention it is preferred that the phosphorylated target molecules not be separated before analysis.

When starting with a sample source that is not appropriate for separation, e.g., whole cells or tissue homogenate, the sample needs to first be prepared, using techniques well known to those skilled in the art. Preparation of the sample will depend on how the phosphorylated target molecules are contained in the sample (*see* e.g., Current Protocols in Molecular Biology; Herbert, *Electrophoresis 20*:660-663 (1999)). For example, an optional way of preparing samples for 2-D gel electrophoresis followed by labeling with the compositions and methods of the present invention includes lysing cells using a lysis buffer that ensures that the proteome, in addition to post-translational modifications, of a sample remain in their *in vivo* state throughout the entire procedure. Examples of such buffers include ones derived from a urea/NP-40/2-mercaptoethanol mixture. Therefore, the lysis buffer might additionally

35:150-54 (1956); Dole *et al.*, *J. Biol. Chem. 235*:2595-99 (1960); Bligh *et al.*, *Canadian J.*

Biochem. Physiol. 37:914-17 (1959); Folch *et al.*, *J. Biochem. 226*:497-509 (1957). The Dole *et al.* references describe an extraction method that involves extractions of the sample with an isopropyl alcohol/heptane/sulfuric acid mixture, followed by several heptane extractions. The organic phase is dried with nitrogen for use in subsequent steps. The Folch *et al.* reference describes the extraction of lipids from biological tissue homogenates or body fluids. Samples are extracted with chloroform/methanol, filtered and reverse-extracted with 0.1 M KCl. The Bligh *et al.* reference describes the organic extraction of lipids from biological tissue homogenates or fluids. Samples are extracted with methanol/chloroform and chloroform, and then filtered and reverse-extracted with water.

Typically, the phosphorylated target molecules (proteins, peptides, carbohydrates or lipids) are present on or in a solid or semi-solid matrix. In one aspect of the invention, this matrix comprises an electrophoresis medium, such as a polyacrylamide gel, agarose gel, linear polyacrylamide solution, polyvinyl alcohol gel or a hydrogel. The solid or semi-solid matrix can also comprise a membrane, such as a filter membrane, a nitrocellulose, poly(vinylidene difluoride) (PVDF) membrane, or nylon membrane wherein the phosphorylated target molecules are immobilized on the membrane by blotting, spotting, electroblotting (tank and semi-dry), capillary blotting or other methods of application well known to those skilled in the art. In accordance with the present invention, a solid and semisolid matrix also includes a glass slide, a plastic matrix (e.g., multi-well plate or pin), a glass or polymeric bead or fiber or a semiconductor material. The phosphorylated target molecules may be arrayed on the support in a regular pattern or randomly. A preferred array of the present invention is a hydrogel glass slide support, wherein the phosphorylated target molecules of the sample are arrayed in a regular pattern. The present invention contemplates that the phosphorylated target molecules can be phosphorylated after immobilization on a matrix material, wherein an enzyme substrate is immobilized and the appropriate enzyme and phosphate is incubated with the immobilized substrate. For certain aspects of the invention it is preferred that the phosphorylated target molecules be free from a solid or semi-solid matrix, i.e. not immobilized and present in an aqueous solution as solubilized molecules.

3. Illumination

In a typical detection method, at any time after or during binding with the phosphate-binding compounds of the present invention, the sample is visualized whereby the phosphorylated target maolecule is detected. Visualization can comprise different methods and is dependent on the chemical moiety A that is covalently attached to the metal chelating moiety

elemental analysis of environmental, biological, and pharmaceutical samples. Recently, the

feasibility of directly measuring phosphorous as m/z 31 signal liberated from β-casein using laser ablation ICP-MS has been demonstrated on electroblot membranes (Marshall, P., Heudi, O., Bains, S., Freeman, H., Abou-Shakra, F., and Reardon, K. (2002) "The determination of protein phosphorylation on electrophoresis gel blots by laser ablation inductively coupled plasma-mass spectrometry." *Analyst* 127: 459-461.). Though 16 pmole of the pentaphosphorylated protein was detectable on blots, the technique was not successfully performed on polyacrylamide gels due to very high background signal. This was undoubtedly due to the presence of isobaric species and overlap from adjacent species generated from the polyacrylamide gel matrix and electrophoresis buffer components. The detection of low concentrations of phosphorous presents several analytical challenges for ICP-MS due to its poor ionization in the argon ICP and the presence of interfering polyatomic species directly at mass 31 ($^{15}N^{16}O$ and $^{14}N^{16}O^{1}H$) and indirectly at mass 32 ($^{16}O_2$ and ^{32}S) (Wilbur, S. and McCurdy, E. (2001) "Determination of trace levels of phosphorous in environmental samples with the 7500c ICP-MS system). ICP-MS could be used to detect phosphoproteins stained with the methods of this invention. The detection procedure is envisioned to involve the following steps. First, proteins separated by gel electrophoresis are fixed to remove the SDS. A typical fixative would be 40% methanol/10% acetic acid. Next, gels would be stained for phosphoproteins using the methods of the invention. Next, the gels would be washed to remove excess stain. The more prominent phosphoproteins could be visualized by fluorescence imaging at this point and background staining can be minimized by inspection and adjustment of wash times as appropriate. Gels are then dried down and the gel is subjected to laser ablation ICP-MS by methods similar to those described in Marshall et al, 2002. Sampling can be performed by single or multi-spot analysis, straight line scans or rastering. In the case of rastering, virtual gels can be constructed from the data obtained as described by Loo RR, Cavalcoli JD, VanBogelen RA, Mitchell C, Loo JA, Moldover B, Andrews PC. "Virtual 2-D gel electrophoresis: visualization and analysis of the E. coli proteome by mass spectrometry." *Anal Chem.* 2001 73:4063-70.). Using the ruthenium-containing SYPRO® Ruby dye staining technology, gallium (aluminum or iron) signal from the phosphoprotein stain, as well as ruthenium for the total protein stain could be independently quantified.

Thus, it is contemplated by the present invention that a wide variety of instrumentation may be used to detect the phosphorylated target molecules, e.g., electrospray ionization (ESI) tandem mass spectrometry (MS/MS). A series of different techniques, including automated high performance liquid chromatography (HPLC)-MS/MS, capillary-HPLC-MS/MS, and solid phase extraction (SPE)-capillary zone electrophoresis (CZE)-MS/MS, are described in

target molecules also form part of the invention. Such kits can be prepared from readily

Another kit of the invention that finds use in isolating phosphorylated proteins or peptides from a complex sample mixture comprises a matrix containing phosphate-binding compounds that are covalently attached to the matrix, typically in the form of a column. The kit would typically comprise, in addition to the phosphate-binding compounds immobilized on the matrix, a metal salt, a wash buffer, a moderately acidic binding buffer, and an elution buffer. The metal salt is preferably gallium chloride and the elution buffer preferably comprises barium hydroxide.

Those skilled in the art will appreciate that a wide variety of additional kits and kit components can be prepared according to the present invention, depending upon the intended user of the kit, and the particular needs of the user.

IV. APPLICATIONS

The present invention is useful for a wide variety of applications in a wide variety of areas including, but not limited to, basic research applications, high-throughput screening, proteomics, microarray technology, diagnostics, and medical therapeutics. Those skilled in the art will appreciate that the invention can be used in a wide variety of assay formats in a wide variety of diagnostic applications. The foregoing description seeks merely to illustrate the many applications of the materials and methods of the present invention, and does not seek to limit the metes and bounds of the invention as described in the above sections.

The materials and methods of the present invention are useful for a number of applications. The present invention may be used to generate data that are used as a reference point for a human patient or animal sample for the diagnosis of disease, progression of disease, and/or predisposition for disease. By way of example, if a disease is associated with changes in protein composition in certain cells, e.g., protein phosphorylation in different organ systems, cell sources or tissue types, a patient sample may be used to generate a protein profile according to the materials and methods of the invention, and compared with profiles of corresponding samples of normal or non-diseased samples and/or diseased origin to determine the presence or absence of, progression of, and/or predisposition to the particular disease in question. It is contemplated by the present invention that many diseases may be diagnosed with data or images generated by the materials and methods of the present invention, including diseases for which particular aberrations in protein expression are either known or not known. Such disease states include, but are not limited to, metabolic diseases that are associated with the lack of certain enzymes, proliferative diseases that are

deduced. If no related proteins exist, however, then the amino acid sequence data may be

Lymphoid Protein Database," *Electrophoresis 19*:2004-2009 (1998). Thus, in accordance

with the present invention, cells treated with a suspected drug compound can be compared to untreated cells to generate a 2-D gel electrophoresis profile. Furthermore, it may be observed, for example, that certain drug compounds induce the activation of different sets of kinases or phosphatases. Such evidence could lead to the elucidation of the mechanism by which many drug compounds work and manifest their effects.

A 2-D gel electrophoresis study was performed to generate a phosphoprotein profile in cultures that were subjected to the effect of oxygen/glucose deprivation. The results suggested that this model could be a good method to observe the development of the tissue and its response to an ischaemic lesion. See Tavares *et al.*, "Profile of Phosphoprotein Labeling in Organotypic Slice Cultures of Rat Hippocampus," *Neurochemistry 12*:2705-2709 (2001).

The materials and methods of the present invention can also be used to study biological phenomena, such as, for example, signal transduction, mitosis, cell proliferation, cell motility, cell shape, gene regulation, and many other cellular processes. The mechanism of action of kinases and phosphatases and the physiological relevance of site-specific phosphorylation of substrate proteins can be explored with the materials and methods of the present invention. The materials and methods of the present invention offer the advantage of high-resolution 2-D gel electrophoresis to simultaneously resolve hundreds of cellular polypeptides. Using the materials and methods of the present invention, the potential for the identification of proteins and the expression of their genes at various stages of cell growth, differentiation, or disease, is extensive. Thus, the invention provides methods and materials for the detection and quantitation of phosphorylation of specific cellular proteins that may provide insight into the mechanisms by which phosphorylation is employed for the regulation in cells.

It is well known that the critical events in the cell cycle are controlled by a complex interplay of kinases and phosphatases. Thus, the status of phosphorylation of different protein isoforms during different phases of the life cycle is important to researchers. Thus, in accordance with the materials and methods of the present invention, the phosphorylation of different proteins related to the stage of the cell cycle related to the activity of certain kinases or phosphatases may be explored using the materials and methods of the present invention. By way of example, a global analysis of phosphoproteins in cells can be used to analyze the primary signals of, for example, mitogenesis in selected cells, or in G1 or S phase cells.

Thus, the materials and methods of the present invention may be useful in investigating the phosphorylation status of various proteins during the cell cycle.

Those of skill in the art will recognize that a database can be generated using the materials and methods of the present invention to produce a record that may show the correlation between gene expression at the RNA and protein level to the function of the cell. For examples, in situations where the cells under study are obtained in both cancerous and normal conditions, comparison of the relative gene expression can be used to identify genes that can serve either as diagnostic markers of pathology or as sites for the pharmacological intervention or treatment of, for example, cancer. Similarly, other diseases can be analyzed merely by substituting the source of cells for analysis.

Thus, the present invention may be used to generate a comprehensive phosphoprotein expression profile from any cell type or biological fluid of interest. A cell type of interest may be any cell, or portion thereof with genetic material. A reference cell can be of any cell type in which the difference in protein expression patterns and levels is desired to be measured. Preferably, the cells are maintained as similar to their native state as possible and culture techniques, incubation times etc., are performed identically between the two to minimize any non-naturally occurring differences. For example, development of a comprehensive protein profile of pre-cancerous, and/or malignant test cells and a normal reference cell can be achieved according to the invention. Such expression profiles can be used to characterize molecular events, for example, related to tumor development and the cellular mechanisms involved.

In accordance with the present invention, a cell of interest and a reference cell could be obtained from the same patient to get an individual phosphoprotein expression profile that can be used to diagnose or treat that patient for those diseases that involve protein phosphorylation. For example, when a tumor is excised, a margin of non-transformed cells is typically removed as well. Phosphoprotein expression profiles can help to ensure that the cells removed all had similar profiles to normal cells rather than the metastatic cells from the same patient for those cancers that involve, or are thought to involve, protein phosphorylation.

One example of cell lines that may be analyzed using the materials and methods of the present invention includes human tumor cell lines. For example human tumor cell lines representing a broad spectrum of human tumors and exhibiting acceptable properties and

phosphate from phosphorylated substrates. Thus, the activity of kinases and phosphatases

responsible for phosphorylating and dephosphorylating individual proteins can be analyzed. *See, e.g.*, Fruehling & Longnecker, "In Vitro Assays for the Detection of Protein Tyrosine Phosphorylation and Protein Tyrosine Kinase Activities," *Methods in Mol. Biol. 174(Ch. 36)*:337-343 (2001).

The applications described herein are provided merely to illustrate a wide variety of potential uses of the invention, and are in no way intended to limit the scope of the invention. A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the invention and shall not be construed as being a limitation on the scope of the invention or claims.

EXAMPLES

Generally, the nomenclature as used herein, and the laboratory procedures in cell culture, molecular genetics, and protein chemistry described below are those well known and commonly employed in the art. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. Units, prefixes, and symbols may be denoted in their SI accepted form. Numeric ranges are inclusive of the number defining the range and include each integer within the defined range.

Example 1: Determination of BAPTA selectivity for gallium and galllium ions for phosphorylated target molecules and a screening method for phosphate-binding compounds

(A) BAPTA with trivalent gallium ions selectively detects phosphoproteins.
A comprehensive search of metal-chelating compounds was performed to identify fluorescent reagents that when combined with a gallium salt (gallium chloride) would selectively detect phosphorylated target molecules (particularly phosphopeptides and phosphoproteins) in a mixture of phosphorylated and nonphosphorylated target molecules. The compounds were tested in a fluorescence spectrophotometer for their ability to bind gallium (III) ion and selectively detect the phosphoprotein ovalbumin. Binding to gallium (III) ion was determined by a fluorescence increase of the same compound in the presence of up to 5 μM gallium chloride in 75 mM NaOAc (pH 4.0) and 140 mM NaCl, Ovalbumin detection was also judged by a fluorescence increase; however, the compounds were placed in a solution containing 75 mM NaOAc (pH 4.0), 140 mM NaCl, 1–4 μM ovalbumin, and 0.5 μM

gallium chloride. Selectivity of phosphoprotein detection was evaluated by virtual elimination of the fluorescence increase in the presence of the same solution lacking gallium chloride.

Using compound 1, a variety of metal ions, including iron and gallium, were screened to determine which ion(s) were best suited for phosphoprotein detection. Metal ions were assayed for binding to compound 1, phosphoprotein detection, and general protein staining by monitoring a fluorescence increase at 530 nm in 75 mM NaOAc (pH 4.0), 140 mM NaCl, 0.5–5 μM metal ion, with or without 4 μM ovalbumin or 1 μM lysozyme. Only trivalent cations that bound to compound 1 resulted in a fluorescence increase at 530 nm and only gallium (III) ion was capable of selectively indicating phosphoproteins when bound to compound 1. Therefore, gallium (III) ion is the most preferred metal ion for phosphoprotein detection. This methodology was extrapolated to identify other compounds wherein a different dye waattached to the metal-chelating moiety.

(B) Differential binding affinity of compound 1 for phosphate compounds.
Compound 1 complexed with gallium (III) ion has differential affinities for various phosphate substrates in 75 mM NaOAc (pH 4.0) and 140 mM NaCl. Some of the phosphate-containing compounds studied were inorganic phosphate, phosphate attached to a protein, a peptide or an amino acid, pyrophosphate, ATP, and DNA. The affinities for these phosphate-based substrates for the Compound 1/gallium (III) ion reagent were determined to be ~50 μM for inorganic phosphate and phosphate attached to a protein, a peptide or an amino acid, ~200 nM for pyrophosphate and ATP, and no binding was detected for DNA. Compare these values to the affinity of compound 1 for gallium (III) ion of 2.5 μM. Most known phosphate compounds should fall into one of these three categories with respect to how it will bind to BAPTA gallium (III) ion; 1) single phosphate group (i.e., inorganic phosphate or phosphate on a protein), 2) multiple linked phosphate group (i.e., pyrophosphate or ATP), or 3) bridging phosphate group (i.e. nucleic acids).

(C) Compound 4 displays dual-emission wavelengths upon simultaneously binding to gallium (III) ion and phosphate.
Concentrations of 0.1–1.0 μM of compound 4 in a solution of 75 mM NaOAc (pH 4.0) and 140 mM NaCl display a single emission peak centered at 410 nm (excitation 350 nm). Addition of 10 nM to 1 mM gallium chloride results in a decrease in the 410 nm emission and a concomitant increase in emission at 490 nm, with an isosbestic point of 475 nm. The half-maximal response for this transition from the blue to green emitting state occurs at approximately 1.8 μM gallium chloride. Therefore, 0.1 μM compound 4 with 1.7 μM gallium

chloride in 75 mM NaOAc (pH 4.0) and 140 mM NaCl display both the 410 nm and 490 nm emission peaks. The addition of phosphate can alter the equilibrium between the emission peaks in favor of the longer wavelength 490 nm peak.

(D) Screening for Phosphate-binding compounds that simultaneously bind gallium and immbolized phosphorylated target molecules.

A panel of test proteins was loaded on a denaturing SDS polyacrylamide gel, separated by electrophoresis, and the gels were fixed with 45% methanol, 5 % acetic acid. Typically the test gels contained 500-600 ng each of myosin, β-galactosidase, phosphorylase b, ovalbumin (2 phosphates), carbonic anhydrase, soybean trypsin inhibitor, lysozyme, aprotinin, α_2-macroglobulin, phosphorylase b, glucose oxidase, bovine serum albumin, α_1 acid glycoprotein, carbonic anhydrase, avidin, and lysozyme. The gels also contained a 4-fold dilution series of α-casein (8 phosphates), 500 ng to 2 ng loaded. Thus the gels contained a range of proteins with different physicochemical properties, such as proteins with hydrophobic binding pockets (e.g. BSA), glycosylated proteins (e.g. α_2-macroglobulin, glucose oxidase and avidin), acidic proteins (e.g. soybean trypsin inhibitor), basic proteins (e.g. lysozyme and aprotonin), and two different phosphoproteins (ovalbumin, α-casein). The dilution series of α-casein yielded an estimate of phosphoprotein staining sensitvity. A selection of phosphate-binding compounds comprising different dye labels and different chelating moieties was initially screened in minimal binding buffers of pH 3.0 to 7.0, with a variety of metal ions, in the presence or absence of metal ion. Dye and metal ion concentrations ranged from 0.1 to 10 μM, typically 0.3 to 3 μM, and most frequently at 1.0 μM. Binding conditions that produced preferential staining of phosphoproteins typically were at pH 3.0 to 5.5, in the presence of certain trivalent metal ions. Under these conditions, optimal preferential phosphoprotein staining was obtained with certain dye labels and the BAPTA chelating moiety with an equimolar concentration of Ga^{3+}. Further evaluation of the successful dyes revealed that the pH optimum was 4.0, and that addition of salt (e.g. 250-750 mM NaCl) improved staining specificity chiefly by decreasing intensity of staining of non-phsophoproteins. A broadened screen of dyes was undertaken with 1 μM candidate dye, 1 μM of Ga^{3+} in 50 mM sodium acetate, pH 4.0, 500 mM sodium chloride.

(E) Binding Solution Formulation

The binding solution comprises a phosphate-binding compound with a metal ion in molar ratios of 1:2 to 2:1 and a buffer at about pH 3.0 to 6.0. Typically, the binding solution

comprises a pH 3.0 to 5.5 buffer (50 to 100 mM), salt (e.g. 100 to 1000 mM NaCl, or 100 to 300 mM $MgCl_2$,) and equimolar concentrations of Ga3+ and of phosphate-binding compound (e.g Compound 2), typically 1 to 10 µM each for detection purposes. Concentrations of the metal ion and phosphate-binding compound are typically at leat 100 times higher concentration for isolation purposes than is present in the binding solution for detection purposes (*see*, Example 13). An optimal binding solution for a gel stain was prepared as follows: 500 µg of compound 2 was dissolved in 873 µl water for a 1 mM stock solution. Five g of $GaCl_3$ were dissolved in 28.4 ml water for a 1 M solution, from which 1 ml was combined with 9 ml water to make a 0.1 M solution, from which 10 µl was added to 990 µl water for a 1 mM stock solution. One liter of a 1 M stock solution of sodium acetate, pH 4.0 was prepared by dissolving 136 g of sodium acetate trihydrate in ca. 800 ml water, adjusting pH to 4.0 by adding ca. 23.5 ml 12 N HCl and bringing volume to 1 liter. One liter of a 4 M stock solution of sodium chloride was prepared by dissolving 233.8 NaCl in ca. 800 ml water and bringing the volume to 1 liter with water. The sodium acetate and sodium choride stock solutions were filtered through a 0.45 µm filter. For 100 ml of binding solution, 5 ml of 1 M sodium acetate, pH 4.0, 12.5 ml of 4 M sodium chloride, and 20 ml of 1,2 propanediol were combined with water to a final volume of 100 ml, to which was added while stirring 100 µl of 1 mM $GaCl_3$ and 100 µl of 1mM Compound 2 to obtain a final binding solution of 1 µM Compound 2, 1 µM Ga3+, 20 % 1,2-propane diol, 500 mM NaCl, 50 mM sodium acetate, pH 4.0.

Example 2: Detection of Phosphoproteins in SDS-Polyacrylamide Gels

Phosphoproteins were separated by SDS-polyacrylamide gel electrophoresis utilizing a 4% T, 2.6 % C stacking gel, pH 6.8 and 13% T, 2.6% C separating gel, and pH 8.8, according to standard procedures. % T is the total monomer concentration (acrylamide + crosslinker) expressed in grams per 100 mL and % C is the percentage crosslinker (e.g., *N,N'*-methylene-bis-acrylamide, *N,N'*-diacryloylpiperazine or other suitable agent). The separating gels were 8 cm wide by 5 cm high and 0.75 cm in thickness. After electrophoresis, the gels were fixed by immersing them in 100 mL 45% methanol and 5% acetic acid for 90 minutes. The gels were washed twice in water for a total of 30 minutes. The gels were then added to a binding solution of the invention (Example 1E) and incubated for 120 minutes at room temperature with gentle orbital shaking, typically 50 rpm. The binding buffer contained 50 mM NaOAc (pH 4.0), 250 mM sodium chloride, 20% v/v 1,2-propanediol, 1 µM gallium chloride. To prepare the binding solution, 120 µL of a 1 mM stock solution of Compound 2

and 120 µL of a 1 mM stock solution of gallium chloride were added to 1080 µL water. This mixture was then added to 59 mL of the binding buffer to yield the binding solution. Alternatively, the phosphate-binding compound and the gallium chloride can be added separately, directly to 60 mL of the binding diluent. Binding solutions that utilize other phosphate-binding compounds of the present invention can be prepared and similarly tested for gel staining. After incubation in binding solution, the gel was washed with 75 mL of 50 mM NaOAc (pH 4.0) and subjected to two washes of 30 minutes each.

For Compound 2 and other dyes that can be excited at 532 nm, images were acquired on a Fujifilm FLA 3000 laser scanner using 532 nm excitation and 580 nm bandpass emission filters. For fluorescent phosphate-binding compounds that absorb in the ultraviolet or at visible wavelengths below 532 nm, excitation was performed using 300 nm and detection was via Roche Lumi-Imager or Fujifilm FLA 3000 laser scanner using 473 nm excitation and 580 nm bandpass emission. The data were displayed using Image Gauge Analysis software. Images of phosphoproteins were displayed as dark bands. Proteins not containing phosphate were not labeled or were very lightly stained relative to the phosphoproteins. When gels were labeled as above but with gallium chloride omitted from the binding solution, phosphoproteins were not selectively stained, and could not be distinguished from background or had very light nonspecific staining. Gels were washed overnight with 50 mM NaOAc (pH 4.0) and images were acquired as above. The background and nonspecific staining was further reduced relative to phosphoprotein staining. Replacement of gallium chloride by other gallium salts gave comparable results with all indicators tested; however, replacement by other metals, including Fe^{3+} and Al^{3+} typically gave inferior results in staining of phosphoproteins.

Fixation of the gels in methanol/acetic acid can be done overnight or the gels can be left in fixative for several days. Other salts can be used instead of sodium chloride, including magnesium chloride, magnesium sulfate, and ammonium sulfate. Sodium chloride concentration is preferably between 100 mM to 1000 mM. If salt is not included in the binding solution, nonspecific staining of nonphosphoproteins is increased. Nonspecific staining is reduced to low levels by extensive washing with ~50 mM NaOAc (pH 4.0). Buffers other than NaOAc may be used, including formate and 2-(*N*-morpholino)ethanesulfonic acid. If 1,2-propanediol is omitted, the background staining of the gel is increased but phosphoproteins are still selectively stained. The most effective pH ranges of the acidic buffers are in the range of 3.0 to 6.0.

morpholino)ethanesulfonic acid (pH 3.0), 1000 mM NaCl, 1 μM compound 2, and 1 μM

gallium chloride. The gels were washed with 50 mL of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 3.0), 1 M NaCl twice for 30 minutes per wash, and then in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 3.0). Images were acquired as described in Example 2.

Example 6: Detection of Phosphoproteins in Two-Dimensional Gels

A human MRC-5 lung fibroblast cell lysate protein mixture (150 µg) was diluted into urea buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% Zwittergent 3–10, 0.8% carrier ampholytes (3–10), 65 mM DTT) and applied on a first dimension IPG strip (3–10 nonlinear, 18 cm). After overnight rehydration, the strips were covered with mineral oil and the proteins were focused for 75,000 volts total. IPG strips were then laid on top of 1 mm thick, 20 cm X 20 cm, 12.5% T, 2.6% C polyacrylamide gels containing 375 mM Tris base, pH 8.8 and SDS-polyacrylamide gel electrophoresis was performed according to standard procedures, except that the cathode electrode buffer was 50 mM Tris, 384 mM glycine, 4% SDS, pH 8.8 while the anode electrode buffer was 25 mM Tris, 192 mM glycine, 2% SDS, pH 8.8. After the second dimension electrophoresis, gels were fixed in 750 mL 45% methanol, 5% acetic acid for 20 hours. Gels were washed twice, 75 minutes per wash, with water and then put in 500 ml staining solution. The staining solution contained 50 mM NaOAc, pH 4.0, 250 mM sodium chloride, 20% v/v 1,2-propanediol, 1 µM compound 2, 1 µM gallium chloride. 500 µL of compound 2, in stock solution at 1 mM and 500 µL of gallium chloride, in stock solution at 1 mM were added to 9 ml water. This mixture was then added to 490 mL of the staining buffer. The gel was incubated for 8 hours in the binding solution; the solution was decanted and the gels were washed with 3 changes of 800 mL 50 mM NaOAc, pH 4.0, 30 to 40 minutes per wash, and then washed overnight in 1 liter 50 mM NaOAc, pH 4.0. Images were acquired on a Fujifilm FLA 3000 laser scanner with 532 nm excitation and 580 nm bandpass emission filter and data displayed using Image Gauge Analysis software. Images of phosphoproteins were displayed as dark spots. Proteins not containing phosphate were not stained or were very lightly stained relative to the phosphoproteins. When gels were stained as above but with $GaCl_3$ omitted from the staining solution phosphoproteins were not selectively stained, and could not be distinguished from background or light nonspecific staining. In addition, staining of phosphoproteins resulted in a trail of spots that correlated with different percentage of phosphorylation of the same protein, i.e., the protein had the same molecular weight but the charge was different due to the addition or removal of a phosphate group. Thus, 2-D gel analysis is a useful tool for identify phosphoproteins using methods of the

present invention and allows for identification of changes in phosphorylation of a single protein.

Example 7: Serial Dichromatic Detection of Phosphoproteins and Total Protein in 2-D Gels.

Electrophoresis and phosphoprotein detection was performed as in Example 6. After detection of the phosphoproteins, the gel was stained with 500 ml SYPRO® Ruby protein gel stain by incubating the gel overnight in the stain, and then washing the gel in 7% acetic acid, 10% methanol for two changes, at 30 minutes each wash. Images were acquired as described in Example 2. Alternatively, the orange signal from the phosphorylated and nonphosphorylated proteins is collected with a standard CCD camera-based imaging system with 300 nm UV light excitation and a 600 nm bandpass filter.

Example 8: Detection of Phosphoproteins Electroblotted to PVDF or Nitrocellulose Membranes

Proteins of interest were separated by SDS-polyacrylamide electrophoresis and transferred to PVDF membrane using standard procedures, and the membrane was allowed to air dry. The PVDF membrane was quickly dipped in 100% methanol, washed with a solution of 40% methanol, 5% acetic acid for 15 minutes, and with two changes of water for 10 minutes each. The blot was then added to a binding solution and incubated for 80 minutes at room temperature with gentle orbital shaking. The binding solution contained 50 mM NaOAc, pH 4.0, 500 mM sodium chloride, 1 µM Compound 1 or Compound 4, and 1 µM gallium chloride. Typically, 60 µL of the phosphate-binding compound, in stock solution at 1mM and 60 µL of gallium chloride, in stock solution at 1 mM were added to 540 µL water. This mixture was then added to 29.5 mL of the staining buffer. Alternatively the phosphate-binding compounds and the gallium chloride may be added separately, directly to 30 mL of the staining diluent. After incubation in staining solution, the gel was washed with 50 mL of 50 mM NaOAc; pH 4.0, 2 washes of 30 to 50 minutes each.

Images were acquired with a standard CCD camera imaging system (BioRad FluorS Max) with a reflective 300 nm UV light source, and a 465 nm bandpass emission filter for Compound 4. Proteins not containing phosphate were not labeled or were very lightly stained relative to the phosphoproteins. When the blot was stained as above but with $GaCl_3$ omitted from the staining solution, phosphoproteins were not selectively stained, and could not be distinguished from background or light nonspecific staining. For imaging with

(undigested) and phosphatase-treated samples loaded pairwise, 1250 ng protein per lane.

Phosphoprotein detection was performed as per Example 2 above, with images taken 90 minutes after labeling and again after overnight washing. An additional gel was labeled as per Example 2 but with no gallium chloride in the binding solution. For the gel labeled with the full binding solution, comparisons of the control, undigested sample proteins showed that the phosphoproteins appeared as dark bands according to the software display and the nonphosphoproteins were not labeled or were only very lightly stained. For the gel labeled with the formulation lacking gallium chloride, phosphoproteins showed the same degree of no labeling or only very light staining as the nonphosphoproteins, and this level of signal was the same as the nonphosphoproteins in the gels labeled with the full formulation including gallium chloride. Comparison of the pairwise phosphoproteins in the fully labeled gel showed that the signal from the alkaline phosphatase–treated sample was significantly less than the signal from the undigested control. The very light signal from the nonphosphoproteins, if detectable, was virtually the same for the control and enzyme-treated samples.

After detection of the phosphoproteins, the gel was stained for total protein with SYPRO® Ruby protein gel stain as per Example 2 and images of SYPRO® Ruby staining were acquired as per Examples 3 and 7. The signal for total protein staining was similar for the pairwise control and digested samples for both gels, indicating that the reduced signal from alkaline phosphatase–treated phosphoprotein samples was not due to protein degradation.

Example 11: Detecting Kinase Activity

Bovine muscle myosin light chain was incubated with commercially available cloned calmodulin-dependent protein kinase II (New England BioLabs) according to the manufacturer's instructions, with 100 mM adenosine triphosphate (ATP) and the supplied buffer components. A parallel, control incubation was done with no enzyme. A sample of each reaction mixture was loaded in adjacent lanes and analyzed by electrophoresis as in Example 2. The gels were fixed in 100 mL of 45% methanol, 5% acetic acid for 60 minutes. The gels were then washed with several changes of water. One gel was incubated for 110 minutes in 30 mL of binding solution containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 3.0, 1000 mM NaCl, 1 µM compound 2, 1 µM gallium chloride. The other gel was incubated in an identical solution, minus gallium chloride. The gels were washed with 50 mL 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 3.0, 1000 mM NaCl twice for 30 minutes per wash, and then in 50 mM 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 3.0. Image acquisition for phosphoprotein detection was done as in Example 2 and serial dichromatic detection of phosphoproteins and total protein was done as in Example 3.

phosphopeptides from the phosphate-binding compound/gallium complex using organic or

35 nm ± 4nm. Alternatively, fluorescence was measured with the ...

Counter (PerkinElmer Life Sciences) using wavelength settings excitation 535 ± 17.5 nm and

emission wavelength setting 590 nm ± 17.5 nm. The binding solution alone and binding solution in the presence of non-phosphorylated peptide demonstrates very similar fluorescence polarization and anisotropies. However, in the presence of the phosphopeptide there is a significant increase in the fluorescence polarization and the anisotropy values. This result demonstrates specific binding of the phosphopeptide to the Compound 2–Ga^{3+}complex in solution but not the non-phosphorylated peptide. *See* Figure 10B.

This assay also provides a method for screening compounds that will bind trivalent gallium ions and label phosphorylated peptides and for solution based kinase assays.

Example 15: Isolation and Characterization of Phosphopeptides from Complex Protein Digests with a Matrix Immobilized Phosphate-binding compound

A phosphate-binding compound-agarose column (compound 13 or 14) (typically 200 µL of medium) was charged with 0.1M $GaCl_3$ and washed with de-ionized H_2O until the pH of the flow-through material approached 7.0. The column was then equilibrated with 5 column volumes of binding buffer (100 mM NaOAc buffer (pH 3.0)). The phosphopeptide mixture was vacuum dried in the SpeedVac (Savant) or similar instrument and dissolved in binding buffer. If the final pH of the peptide mixture is not 3.0, then it can be adjusted with 1–10 M acetic acid as appropriate. The protein digest (1–5 mg/ml) was applied in 1 column volume or less (but no less than half the column volume) and followed with 2 column volumes of binding buffer. Flow-through (FT) fractions were combined and stored for further analysis. The column was washed with 2 column volumes of 100 mM NaOAc (pH 7.0), 500 mM NaCl, 10% acetonitrile followed by 1 column volume of NaOAc (pH 7.0). The FT fractions were combined and stored for further analysis. Bound peptides were eluted with 3 separate column volumes of saturated $Ba(OH)_2$ that are collected in a single tube. The pH of the resulting elution fraction was greater than pH 11.0, and when it was not, it was immediately adjusted with saturated barium hydroxide ($Ba(OH)_2$). The elution fraction was incubated for 90 minutes at 30°C. After incubation, the sample was divided into 2 portions, one of which was neutralized to pH 5.0–7.0 with glacial acetic acid and stored frozen. One half volume of de-ionized water is added to the other tube followed by the addition of a concentrated nucleophilic thiol or amine (methylamine, cystamine or β-mercaptoethylamine) to achieve a final concentration of 0.1–0.5 M in a volume not exceeding 1/6 of the starting sample/H_2O volume. The reaction mix was incubated for an additional 60 minutes at 30°C, then neutralized to pH 5.0–7.0 with glacial acetic acid. For MALDI-TOF mass spectrometry analysis, peptides were purified from samples using C18 ZipTips (Millipore) using standard

protocols, vacuum dried in a SpeedVac dryer and dissolved in 50% acetonitrile and 0.1% TFA. An equal volume of 10 mg/mL MALDI matrix (α-cyano-5-hydroxycinnamic acid) in the same solvent was added. The solution was mixed thoroughly and 1 μL is spotted onto the MALDI target.

Differential mass weight analyses of both peptide fractions resulted in the determination of the number of phosphorylation sites on the peptides, as well as the nature of the phosphoamino acids. Under the conditions used, only phosphoserine residues undergo elimination and nucleophilic addition (loss of phosphoric acid -98 amu, + mass weight of nucleophilic addition reagent). Phosphothreonine residues undergo elimination only (loss of phosphoric acid only, -98 amu) and phosphotyrosine residues remain unchanged, as phosphotyrosine is stable in strong base.

Example 16: Quantitating the Number of Phosphates on Ovalbumin.

Solutions of 1 μM and 4 μM ovalbumin were incubated in 75 mM NaOAc (pH 4.0), 140 mM NaCl, 0.1 μM Compound 4, and 1.7 μM gallium chloride at room temperature for 5–10 minutes. The fluorescence intensity of the resulting solution was then measured at 410 nm in a fluorescence spectrophotometer and compared to a standard phosphate calibration curve to determine the number of phosphates on ovalbumin. The standard phosphate calibration curve was produced by equilibrating known concentrations (1, 2, 4, 6, 8, and 10 μM) of a 19 amino acid phosphoserine-containing peptide in 75 mM NaOAc (pH 4.0), 140 mM NaCl, 0.1 μM compound 4, and 1.7 μM gallium chloride and measuring the fluorescence intensity at 410 nm. Next the fluorescence intensity was graphed versus the known concentration of phosphopeptide. The fluorescence intensity from the solution containing ovalbumin was compared to the standard curve to reveal ~2 μM and ~8 μM phosphate. Finally, accounting for the protein's concentration resulted in the determination of two phosphate groups per molecule of ovalbumin.

Example 17: Phospholipid Detection

To test the detection of phospholipids with the present invention, different phospholipids were spotted onto a nitrocellulose membrane. The phospholipids were obtained from Echelon Research Labs in a format called a PIP Array™, which contains 8 different phosphoinositides (PtdIns) at 7 different concentrations. PIP Arrays™ were used for determining the sensitivity limits of the invention for detecting phospholipids.

PIP Array™

1. PtdIns 100 50 25 12.5 6.3 3.2 1.6 pmol
2. PtdIns (3) P
3. PtdIns (4) P
4. PtdIns (5) P
5. PtdIns (3,5) P2
6. PtdIns (4,5) P2
7. PtdIns (3,4) P2
8. PtdIns (3,4,5,) P3

One PIP Array™ was washed in 50 mM NaOAc (pH 4.0) for 15 min. After the wash, the PIP Array™ was incubated in 50 mM NaOAc (pH 4.0), 20% 1,2-propanediol, 500 mM NaCl, 1 µM compound 1, and 1 µM $GaCl_3$ for 1 hour, by incubating the array at 100–150 RPM on an orbital shaker. After incubating the PIP Array™ the array was washed 3 times in 50 mM NaOAc (pH 4.0) for 15 minutes each at 100–150 RPM on an orbital shaker to remove unbound dye and reduce the background fluorescence. An image of the PIP Array™ was generated using a Laser scanner (Fuji FLA 3000) with an excitation wavelength of 473 nm and an emission filter of 520 nm. Of the eight phosphoinositides, four gave a strong positive signal. These included phosphatidic acid, phosphoinositide (4,5) P_2, phosphoinositide (3,4) P_2 and phosphoinositide (3,4,5) P_3. The strongest signal was obtained with phosphoinositide (3,4) P_2 followed by phosphoinositide (4,5) P_2 and then phosphoinositide (3,4,5) P_3.

Example 18: Phosphoprotein Detection on Microarrays

Four specific, purified proteins including β-casein, ovalbumin, pepsin and bovine serum albumin were arrayed from a source plate (384 well plate) at a concentration of 0.975 µg/mL

NaOAc, pH 4.0, containing 10% methanol followed by a 15-minute water wash. Slides were then spun briefly in a microarray high-speed centrifuge affixed with a rotor with a slide holder

(Telechem) at ~ 6000 rpm to remove excess liquid. After the slides were dry, the arrays were imaged using the ScanArray® 5000 XL Microarray Analysis System (Packard Instrument Co., Meriden, CT) using the 543.5 nm laser and either 570 nm or 592 nm emission filter.

Example 20: Detection of Immobilized Kinase Substrates in Microarray Format; Selective Detection of Glycogen Synthase 1–10.

Two specific peptides, Abl peptide and glycogen synthase 1-10, were arrayed from a source plate (384-well plate) concentration of 0.03 – 2 mg/ml in water, onto HydroGel coated slides (Perkin Elmer). Abl peptide (New England Biolabs) is a substrate for Abl tyrosine kinase and its amino acid sequence is E-A-I-Y-A-A-P-F-A-K-K-K (MW 1336). Glycogen synthase 1–10 (Calbiochem) is a substrate for Calcium-Calmodulin-Dependent protein Kinase II and its amino acid sequence is P-L-S-R-T-L-S-V-S-S (MW 1045.2). Arrays were spotted using a manual glass slide arrayer (V&P Scientific, San Diego, CA) fixed with 4 rows of 8 pins (32 total), ~500 micron diameter spot size, 1.125 micron horizontal pitch and 750 micron vertical pitch (pitch = center-to-center spacing of spots). The handarrayer collected 6 nL of peptide from the source plate and transferred ~6 nL to the hydrogel coated slide by direct contact. The resultant peptide concentration is 0.18 to 12 ng/spot. Peptides were arrayed in replicates of 6, resulting in array of 96 spots (12 spots, of which were 0 ng/spot). Slides were left overnight after arraying in a humidity chamber. Slides were then blocked for 1 hour in 100 mM HEPES, 1% BSA while rotating (Barnstead/Thermolyne Labquake rotisserie). After blocking, the slides were spun briefly in a small microarray high-speed (max ~6000 rpm) centrifuge affixed with a rotor with a slide holder (Telechem) to remove excess liquid. Next, kinase reactions were performed by attaching a Grace Biolabs Hybriwell™ hybridization sealing system (40 x 22 x 0.25 mm) to the hydrogel coated slide to enclose the area containing the hydrogel polyacrylamide pad. The reaction was carried out in an 80 µL reaction volume containing 20,000 U/mL or 1600 units enzyme (Calmodulin-Dependent protein Kinase II, NEB) using buffer, $CaCl_2$, calmodulin, and ATP supplied with the enzyme. 1X CamKII buffer included 50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM Na_2EDTA, pH 7.5. $CaCl_2$, calmodulin and ATP working concentrations were 2 mM, 1.2 µM and 0.10 mM. The reaction solution with enzyme was pipetted into the Hybriwell™ through 1 of 2 ports on the seal cover. Ports were then sealed with seal-tabs, placed in a CMT-hybridization chamber (VWR Scientific) and incubated on a nutator (Clay Adams) in a 37°C incubator. The kinase reaction was carried out for 3 hours. After incubation, the slides were removed from the hybridization chamber and washed 2 times for 5 minutes in 10% SDS

(phosphoprotein and total protein). The ratio of these fluorescence intensities was then

place of ATP at a working concentration of 0.10 mM. A control reaction was run
simultaneously on a second slide using ATP itself, supplied with the enzyme, at a working

35 elimination/validation

a rotisserie in a binding solution comprising [illegible]

NaCl, 20% propanediol, 1 µM $GaCl_3$, and 0.05 M sodium acetate, pH 4.0. Slides were then

washed three times for 15 minutes on a rotisserie in 0.05 M sodium acetate, pH 4.0, containing 4% acetonitrile followed by a 15 minute water wash. Slides were then spun briefly in a microarray high-speed centrifuge affixed with a rotor with a slide holder (Telechem) at ~ 6000 rpm to remove excess liquid. After slides were dry, the arrays were imaged using the ScanArray® 5000 XL Microarray Analysis System (Packard Instrument Co., Meriden, CT) using the 543.5 nm laser and 570 nm emission filter. The binding solution specifically labeled 1.3 - 2.6 pg pDSIP, 2.6 – 5.2 pg pKemptide and 10.4 – 20.8 pg pp60 c-src (pY). Following phosphopeptide detection, the slides were immediately placed in blocking buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Tween-20, 0.25% MOWIOL-488, 0.5% BSA and incubated while rotating for 3 – 5 hours. Slides were then transferred to blocking buffer (described above) containing a 1:1000 dilution (final concentration of 1.5 µg/ml) of phospho-tyrosine monoclonal antibody (supplied at 1.5 mg/ml; P-Tyr-100; Cell Signaling Tech.) and incubated overnight at 4°C while rotating. After overnight incubation with the primary antibody, the slides were washed three times for 10 minutes in blocking buffer and then incubated for 45 minutes, while rotating, in blocking buffer containing a 1:5000 dilution (final concentration of 0.4 µg/ml) of Alexa Fluor® 647 goat anti-mouse (supplied at 2 mg/ml). Finally, slides were washed two times for 10 minutes in blocking buffer followed by two 5 minute washes in 50 mM Tris, pH 7.5, 150 mM NaCl and spun briefly in a microarray high-speed centrifuge. After the slides were dry, the arrays were imaged using the ScanArray® 5000 XL Microarray Analysis System (Packard Instrument Co., Meriden, CT) using two protocols with a 543.5 nm laser/570 nm emission filter set and a 632.8 nm laser/670 nm emission filter set. Using the 543.5 nm excitation and 570 nm emission filter, there was no signal detected. Using the 632.8 nm excitation and 670 nm emission filter, pp60 c-src (pY) was specifically detected to a sensitivity of 5.2 pg.

(B) After overnight incubation with the primary antibody, the slides were washed three times for 10 minutes in blocking buffer and then incubated for 45 minutes, while rotating, in blocking buffer containing a 1:100 dilution (final concentration of 2 µg/ml) of Zenon™ One Alexa Fluor® 647 mouse IgG labeling reagent (supplied at 200 µg/ml). Finally, slides were washed once for 5 minutes in blocking buffer, once for 5 minutes in 50 mM Tris, pH 7.5, 150 mM NaCl and spun briefly in a microarray high-speed centrifuge. After the slides were dry, the arrays were imaged using the ScanArray® 5000 XL Microarray Analysis System (Packard Instrument Co., Meriden, CT) using two protocols with a 543.5 nm laser/570 nm emission filter set and a 632.8 nm laser/670 nm emission filter set. Using the 543.5 nm excitation and 570 nm emission filter, there was no signal detected. Using the 632.8 nm

temperature then evaporated. The residue was purified by

chloroform/methanol/acetic acid (50:5:1) to give 3.0 g of X-Rhod-5F tetramethyl ester as a red foam.

To X-Rhod-5F tetramethyl ester (3.0 g, 2.9 mmol) in dioxane (25 mL) and methanol (25 mL) was added 1 M KOH (30 mL, 30 mmol). The solution was stirred overnight then evaporated. The residue was dissolved in 10 mL water and this added to 50 mL of 5% HCl. A precipitate was filtered and dried to give 500 mg of Compound 5 free acid as a purple powder. 100 mg of the free acid was converted to the potassium salt with aqueous KOH, followed by chromatography with water on Sephadex LH-20 to give 40 mg of Compound 5 as its potassium salt, a purple powder.

Example 30: Synthesis of Quinazolinone-Labeled BAPTA (Q-BAPTA) Compounds (Compounds 7 and 23)

Preparation of 5-Fluoro-Q-BAPTA (Compound 7): a catalytic quantity of *p*-toluenesulfonic acid (TsOH) was added to a solution of anthranilamide (29 mg, 0.21 mmol) and 5'-fluoro-5-formyl-4-hydroxy-BAPTA tetramethylester (128 mg, 0.21 mmol) in 10 mL dichloroethane/5 mL ethanol. The solution was refluxed overnight then cooled. Chloranil (57 mg, 0.23 mmol) was added. After 2 hours, the solution was evaporated and the residue was purified by flash chromatography using 5% methanol/chloroform to yield 50 mg of the tetramethylester of Compound 7 as a light-amber immobile oil; m/z 711 (710 calc for $C_{34}H_{34}N_4O_{12}F$).

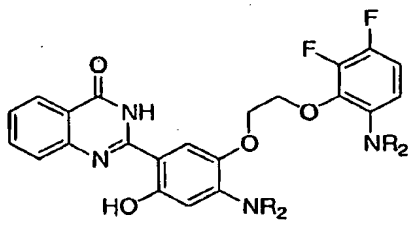
To a green solution of the tetramethylester of compound 7 (50 mg, 0.07 mmol) in 1:1 dioxane:methanol (5 mL), was added 1 M aqueous KOH (0.56 mL, 0.56 mmol). The yellow solution was stirred overnight then evaporated. The residue was purified with water on Sephadex LH-20, generating 53 mg of compound 7 as its potassium salt as a yellow powder; m/z (positive mode) 655 (651 calculated for $C_{30}H_{23}N_4O_{12}F$).

Preparation of 5,6-Difluoro-Q-BAPTA (Compound 23): 5,6-Difluoro-4'-hydroxy-5'-formyl BAPTA tetramethylester (0.100 g, 0.163 mmol) and anthranilamide (0.022 g, 0.162 mmol) were dissolved in a mixture of methylene chloride (10 mL) and ethanol (5 mL). TsOH (5 mg) was added and the reaction mixture was refluxed for 3 hrs. Chloranil (0.044 g, 0.18 mmol) was added to the solution. The mixture was refluxed for 2 more hours and evaporated. The crude product was purified by preparative TLC using 2:1 chloroform-ethyl acetate as eluant. The main component (R_f = 0.5) was isolated with ethyl acetate, which solution was

evaporated to give 5,6-difluoro-Q-BAPTA tetramethylester as a colorless powder (0.029 g, 24%).

5,6-Difluoro-Q-BAPTA tetramethylester (0.027 g, 0.037 mmol) was dissolved in a mixture of 1 mL of methanol and 1 mL of dioxane. 1 M KOH (1 mL) was added to the solution and the reaction mixture was kept overnight at room temperature. Volatiles were evaporated, the crude product was redissolved in water and purified on a Sephadex LH-20 column, eluting with water. The product was lyophilized to give 0.021 g of 5,6-difluoro-Q-BAPTA potassium salt (Compound 23) as a yellow powder (R=CH_2CO_2K).

Compound 23



Example 31: Synthesis of (BODIPY-BAPTA) Compounds (Compound 8 and 24)

Preparation of BODIPY FL BAPTA-5F (Compound 8): To a cold solution of 5-fluoro-BAPTA tetramethylester (1.00 g, 1.82 mmol) in 9 mL acetic anhydride was added 70% nitric acid (0.15 mL, 2.3 mmol). After 10 minutes, the reaction solution was poured into 30 mL aqueous NaOAc then saturated aqueous sodium bicarbonate was added. The mixture was extracted with chloroform (2 x 30 mL). The extract was washed with brine, dried over sodium sulfate, and concentrated to an amber residue. This was purified by flash chromatography using ethyl acetate/hexanes to give 0.43 g of 5-nitro-5'-fluoro-BAPTA, tetramethylester as a yellow powder.

To 5-nitro-5'-fluoro-BAPTA, tetramethylester (0.43 g, 0.72 mmol) in 1:1 methanol/dioxane (10 mL) was added 1 M KOH (5.8 mL, 5.8 mmol). The solution was stirred overnight then evaporated. The residue was dissolved in 10 mL water, and the pH lowered to 2 with aqueous HCl. A precipitate was collected and dried to give 0.31 g of 5-nitro-5'-fluoro-BAPTA free acid as a yellow powder.

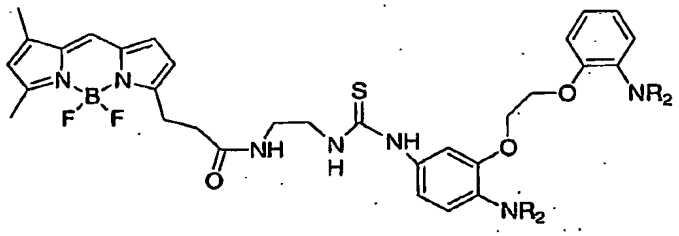
A solution of 5-nitro-5'-fluoro-BAPTA free acid (0.31 g, 0.58 mmol) in 30 mL methanol was shaken over 10% Pd/carbon (0.15 g) under 38 psi hydrogen gas for 6 hours, then filtered and evaporated to give 0.26 g of 5-amino-5'-fluoro-BAPTA free acid as a colorless powder.

BODIPY FL free acid (Molecular Probes, Inc. D-2183, 27 mg, 0.09 mmol) in 5 mL anhydrous THF was treated with an oxalyl chloride (0.20 mmol) and diisopropylethylamine (DIEA, 0.20 mmol) under argon. After 15 minutes, the solution was evaporated. The residue was dissolved in 3 mL anhydrous dioxane, and this solution was slowly added to a solution of 5-amino-5'-fluoro-BAPTA free acid (50 mg, 0.10 mmol) in 5 mL water that had been pH-adjusted to pH = 9.5 with sodium carbonate. This solution was stirred overnight then evaporated to near dryness. This solution was purified with water elution on Sephadex LH-20 to yield 41 mg of BODIPY FL BAPTA-5F (Compound 8), sodium salt as an orange powder.

Preparation of BODIPY FL-EDA-BAPTA (Compound 24)

The pH of a solution of BODIPY FL ethylenediamine hydrochloride salt (15 mg, 0.04 mmol, Molecular Probes) in 3 mL water was raised to 7.6 by dropwise addition of aqueous sodium bicarbonate. A solution of 5-isothiocyanato-BAPTA free acid (22 mg, 0.04 mmol) in 2 mL dioxane was added. The pH was raised to 9.5 with aqueous sodium carbonate, and the orange solution was stirred at room temperature overnight. The solution was evaporated to 2 mL, and the this solution was purified on Sephadex LH-20 using water for elution to give 17 mg of BODIPY FL-EDA-BAPTA sodium salt (Compound 24) as a fine orange powder after lyophilization (R=CH_2CO_2Na).

Compound 24



Example 32: Synthesis of biotinylated BAPTA compounds (Compounds 9, 12 and 18)

Preparation of Biotin-BAPTA-5F (compound 12)

A solution of 5-nitro-5'-fluoro-BAPTA, tetramethylester was reduced by catalytic hydrogenation over 10% Pd/C in ethyl acetate. To the resulting 5-amino-5'-fluoro-BAPTA, tetramethylester (0.10 g, 0.18 mmol) in anhydrous dichloromethane/THF (4:1, 5 mL) was added glutaric anhydride (40 mg, 0.36 mmol) and catalytic DMAP. The solution was stirred overnight then evaporated. The residue was purified by flash chromatography using 10% methanol/chloroform to give 0.13 g of the glutaramide of 5-amino-5'-fluoro-BAPTA, tetramethylester as an oil.

To the glutaramide of 5-amino-5'-fluoro-BAPTA, tetramethylester (0.18 mmol) in 5 mL anhydrous THF and 5 mL anhydrous acetonitrile was added *N*-hydroxysuccinimidyluronium tetrafluoroborate (108 mg, 0.36 mmol). After two hours a solution of biotin ethylenediamine hydrobromide (66 mg, 0.18 mmol, Molecular Probes) and DIEA (0.05 mL) in 2 mL anhydrous DMF was added. After stirring overnight, the volatiles were evaporated. The residue was triturated with water (15 mL), and the resulting precipitate was collected, rinsed with water, and dried to give 0.10 g of Biotin-BAPTA-5F tetramethylester as a gray powder.

To Biotin-BAPTA-5F tetramethylester (0.10 g, 0.11 mmol) in 1:1 methanol/dioxane (4 mL) was added 1 M KOH (1.0 mL, 1.0 mmol). The solution was stirred overnight then evaporated. The residue was purified on Sephadex LH-20 using water, which gave Biotin-BAPTA-5F (Compound 12) potassium salt as a colorless powder after lyophilization.

Preparation of Rhod-biocytin (Compound 18)

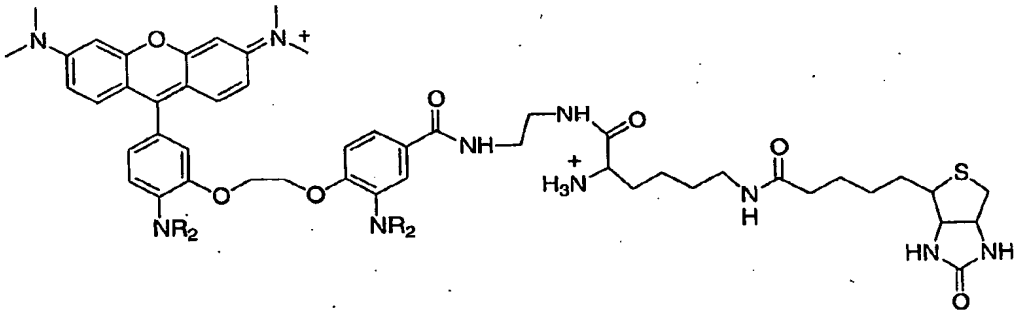
To a 0.5 M solution of 4-(succinimidyloxycarbonyl)-rhod tetramethyl ester in anhydrous THF was added 1.1 equivalent of *N-t*-BOC-ethylenediamine and 1.1 equivalent of DIEA. The resulting solution was stirred for 30 minutes then evaporated. The residue was purified by flash chromatography using chloroform/methanol/acetic acid. The purified carbonate was dissolved in dichloromethane and treated with trifluoroacetic acid (20 equivalents). This solution was stirred 30 minutes, then evaporated and dried to give the ethylenediamine carboxamide of 4-carboxy-rhod tetramethyl ester.

To a 0.5 M solution of the ethylenediamine carboxamide of 4-carboxy-rhod tetramethyl ester in DMF was added *N-t*-BOC-biocytin succinimidyl ester (1.5 equivalent, described in Wilbur et al., *Bioconjugate Chemistry* **2000**, 11: 584-98) and DIEA (1.5 equivalent). The resulting solution was stirred at room temperature until the TLC indicated consumption of the

fluorescent starting material. The volatiles were removed *in vacuo*, and the residue was purified by flash chromatography using chloroform/methanol/acetic acid to give *N*-*t*-BOC-rhod-biocytin tetramethyl ester.

A 0.5 M solution of *N*-*t*-BOC-rhod-biocytin tetramethyl ester in 1:1 methanol/dioxane was treated with 12 equivalents of 1 M KOH. The resulting solution was stirred overnight at room temperature then evaporated to dryness. The residue was purified on Sephadex LH-20 using water to give Compound 18 as a red powder after lyophilization (R=CH_2CO_2K).

Compound 18



Preparation of Rhod-4-biotin-BAPTA (Compound 9)

A suspension of (2'-nitrophenoxy)-2-chloroethane (20.15 g, 0.10 mol), methyl (4-hydroxy-3-nitro)benzoate (21.67 g, 0.11 mol), and K_2CO_3 (27.60 g, 0.20 mol) was stirred at 130 °C for 16 h, cooled to room temperature, and poured into ice water (1.2 L). The precipitate was filtered, washed with H_2O and dried to give 32.00 g of (4'-methoxycarbonyl-2'-nitrophenoxy)-2-(2"-nitrophenoxy)ethane as a yellow solid. 4'-Methoxycarbonyl-2'-nitrophenoxy)-2-(2"-nitrophenoxy)ethane (20.0 g, 55.2 mmol) was hydrogenated over 10% Pd/C (3.0 g) in DMF (300 mL) at 40 psi for 5 h. The mixture was filtered from catalyst through Celite. The filtrate was evaporated and ether (100 mL) was added. The product was filtered and washed with ether (2 x 25 mL) to give 13.2 g of 1'-amino-4'-methoxycarbonylphenoxy)-2-(2"-aminophenoxy)ethane as an off-white solid.

A mixture of 2'-amino-4'-methoxycarbonylphenoxy)-2-(2"-aminophenoxy)ethane (13.20 g, 44 mmol), methanol (50 mL), dioxane (50 mL), and 1 M KOH (100 mL, 100 mmol) was stirred at 65 °C for 5 h, then overnight at room temperature. The mixture was evaporated and the

35 (40 mL) was stirred at 68 °C for 20 h, then cooled to room temperature and

NaOAc (600 mL). After 1 h, the precipitate was filtered, washed with water, and dried to give 3.70 g of 4-diphenylmethoxycarbonyl-dihydrorhod tetramethyl ester as a purple-red solid.

A mixture of 4-diphenylmethoxycarbonyl-dihydrorhod tetramethylester (2.050 g, 2.0 mmol) and powdered chloranil (0.492 g, 2.0 mmol) in $CHCl_3$ and MeOH (40 mL of each) was stirred for 2 h, filtered and evaporated. The residue was purified by flash chromatography on SiO_2 using a gradient 5–6.5% MeOH in $CHCl_3$/ 1% AcOH as eluant to give a crude product, which was re-dissolved in $CHCl_3$, filtered from SiO_2, and evaporated to give 0.533 g of 4-diphenylmethoxycarbonyl-rhod tetramethyl ester as a dark-purple solid.

To 4-diphenylmethoxycarbonyl-rhod tetramethyl ester (51 mg, 0.05 mmol) in dioxane (2 mL) and MeOH (1 mL) was added 1 M KOH to give pH 12.0. The mixture was stirred for 20 h, then the pH adjusted to 9.0 with 0.1 M HCl. The mixture was evaporated and the residue purified on Sephadex LH-20 using H_2O as eluant. The product was lyophilized to give 26 mg of 4-carboxy-rhod tetrapotassium salt as a red-purple solid.

To 4-diphenylmethoxycarbonyl-rhod tetramethyl ester (102 mg, 0.1 mmol) in $CHCl_3$ (10 mL) was added TFA (10 mL) and the resulting mixture was stirred for 1 h then evaporated and co-evaporated with $CHCl_3$ (3 x 10 mL). Ether (10 mL) was added and the precipitate was filtered and washed with ether (3 x 10 mL) to give 82 mg of 4-carboxy-rhod tetramethyl ester as a dark purple solid.

To 4-carboxy-rhod tetramethyl ester (80 mg, 0.093 mmol) in DMF (2 mL) was added DIEA (0.35 mL, 2 mmol) and dry *O*-trifluoroacetyl-*N*-hydroxysuccinimide (TFA-SE, 225 mg, 1 mmol). The mixture was stirred for 2 h, then more TFA-SE (113 mg, 0.5 mmol) was introduced and the mixture stirred for another 16 h. The mixture was diluted with $CHCl_3$ (50 mL), washed with 1% AcOH (3 x 20 mL), H_2O (25 mL), sat. NaCl (50 mL), filtered and evaporated. Ether (25 mL) was added and the precipitated product was filtered and washed with ether to give 86 mg of 4-(succinimidyloxycarbonyl)-rhod tetramethyl ester as a dark-purple solid.

To biotin cadaverine (34 mg, 0.077 mmol, Molecular Probes, Inc.) in DMF (1 mL) and DIEA (0.055 mL, 0.40 mmol) was added a solution of 4-(succinimidyloxycarbonyl)-rhod tetramethyl ester (36 mg, 0.038 mmol). The mixture was stirred for 3 h, diluted with $CHCl_3$ (200 mL), washed with 1% AcOH (3 x 150 mL), H_2O (100 mL), sat. NaCl (200 mL), filtered and evaporated. The residue was purified on two preparative TLC SiO_2 plates, using 12% MeOH

and 2.5% AcOH in $CHCl_3$ as eluant to give 38 mg of 4-(*N*-(5"-biotinylaminopentane)aminocarbonyl)-rhod tetramethyl ester.

To 4-(*N*-(5"-biotinylaminopentane)aminocarbonyl)-rhod tetramethyl ester (30 mg, 0.025 mmol) in MeOH (2 mL) and H_2O (1 mL) was added 1 M KOH to give pH 12.0. The mixture was stirred for 20 h then adjusted to pH 8.5 with 0.1 M HCl. The mixture was evaporated and the residue purified on Sephadex LH-20 using H_2O as eluant. The product was lyophilized to give 30 mg of Compound 9 (4-(*N*-(5"-biotinylaminopentane)aminocarbonyl)-rhod tetrapotassium salt) as an orange-red solid.

Example 33: Synthesis of 4-(4'-(Aminophenyl)-2-ethylamino)carbonylmethyl-rhod tripotassium Salt (Compound 10).

A suspension of (2'-nitrophenoxy)-2-chloroethane (5.87 g, 29 mmol), methyl 4-hydroxy-3-nitrophenyl acetate (6.15 g, 29 mmol), and K_2CO_3 (8.28 g, 60 mmol) was stirred at 120 °C for 16 h, cooled to room temperature, and poured into ice water (0.6 L). The precipitate was filtered, washed with H_2O and dried to give 4.49 g of (4'-methoxycarbonylmethyl-2'-nitrophenoxy)-2-(2"-nitrophenoxy)ethane as a yellow solid.

4'-(Methoxycarbonylmethyl-2'-nitrophenoxy)-2-(2"-nitrophenoxy)ethane (9.6 g, 25.5 mmol) was hydrogenated over 10% Pd/C (1.0 g) in DMF (250 mL) at 40 psi for 16 h. The mixture was filtered from catalyst through Celite. The filtrate was evaporated and the residue was purified by flash chromatography on SiO_2 using a gradient of 25–35% EtOAc in hexanes to give 5.53 g of (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2"-aminophenoxy)ethane as an off-white solid.

A mixture of (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2"-aminophenoxy)ethane (5.50 g, 17.4 mmol), methanol (40 mL), dioxane (40 mL), and 1 M KOH (35 mL, 35 mmol) was stirred at 45 °C for 1 h, then overnight at room temperature. The mixture was evaporated and the residue was suspended in H_2O (100 mL). Aqueous 1 M HCl was added to pH 3.0. Precipitated product was filtered, washed with H_2O, and dried to give 4.59 g of (2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2"-aminophenoxy)ethane as an off-white solid.

Diphenyldiazomethane was prepared by vigorously stirring benzophenone hydrazone (2.94 g, 15 mmol) and yellow HgO (8.80 g, 40 mmol) in hexanes (70 mL) for 5 h. The mixture was filtered from inorganics, and the filtrate was evaporated and the residue was redissolved in

acetone (20 mL). This solution was added to the solution of the 2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2''-aminophenoxy)ethane (3.02 g, 10 mmol) in acetone (120 mL). The resulting mixture was stirred for 16 h at 35 °C, then the excess of diphenyldiazomethane was decomposed with AcOH (0.5 mL) over 2 h. The mixture was evaporated, and the crude product was purified by flash chromatography on SiO_2 using 1% MeOH in $CHCl_3$ as eluant to give 4.44 g of (2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2''-aminophenoxy)ethane as an off-white solid.

A mixture of 2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2''-aminophenoxy)ethane (2.12 g, 4.5 mmol), DIEA (4.0 mL, 23.5 mmol), methyl bromoacetate (8.8 mL, 94 mmol), and NaI (0.50 g, 4.7 mmol) in MeCN (90 mL) was refluxed for 70 h, cooled to room temperature and evaporated. The residue was dissolved in $CHCl_3$ (500 mL), washed with 1% AcOH (3 x 200 mL), H_2O (200 mL), sat. NaCl (200 mL), filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column using a gradient of 30–40% EtOAc in hexanes as eluant to give 2.82 g of 4-diphenylmethoxycarbonylmethyl-BAPTA tetramethyl ester as a colorless solid.

To a solution of Vilsmeier reagent made from $POCl_3$ (1.5 mL, 30 mmol) in DMF (10 mL) was added a solution of 4-(diphenylmethoxycarbonylmethyl)-BAPTA tetramethyl ester (3.78 g, 5 mmol) in DMF (5 mL). The mixture was stirred for 24 h then quickly poured into an ice–sat. K_2CO_3 mixture (500 mL). The mixture was extracted with $CHCl_3$, dried over $MgSO_4$ and evaporated. The mixture of products was separated on SiO_2 using a gradient of 30–40% EtOAc in hexanes to give 1.65 g of aldehyde 4-(diphenylmethoxycarbonylmethyl)-5'-formyl-BAPTA tetramethyl ester as a colorless solid.

A mixture of aldehyde 4-(diphenylmethoxycarbonylmethyl)-5'-formyl-BAPTA tetramethyl ester (784 mg, 1.0 mmol), *m*-dimethylaminophenol (301 mg, 2.2 mmol), and TsOH (20 mg, catalyst) in propionic acid (10 mL) was stirred at 65 °C for 20 h, then cooled to room temperature and poured into 3 M NaOAc (150 mL). After 1 h, the precipitate was filtered, washed with water, and dried to give 450 mg of 4-(diphenylmethoxycarbonylmethyl)-dihydrorhod tetramethyl ester as a purple-red solid.

A mixture of 4-(diphenylmethoxycarbonylmethyl)-dihydrorhod tetramethyl ester (420 mg, 0.43 mmol) and powdered chloranil (122 mg, 0.5 mmol) in $CHCl_3$ and MeOH (20 mL of each) was stirred for 3 h, filtered and evaporated. The residue was purified by flash chromatography on SiO_2 using a gradient of 5–7% MeOH in $CHCl_3$/ 0.5% AcOH as eluant to

give a crude product, which was redissolved in $CHCl_3$, filtered from SiO_2, and evaporated to give 275 mg of 4-(diphenylmethoxycarbonylmethyl-5'-tetramethyl)-rhod tetramethyl ester as a dark-purple solid.

To a solution of 4-(diphenylmethoxycarbonylmethyl-5'-tetramethyl)-rhod tetramethyl ester (250 mg, 0.25 mmol) in $CHCl_3$ (20 mL) was added TFA (20 mL) and the resulting mixture was stirred for 1 h, then evaporated and co-evaporated with $CHCl_3$ (3 x 30 mL). Ether (30 ml) was added to the residue and the precipitate was filtered and washed with ether (3 x 10 mL) to give 200 mg of 4-carboxymethyl-rhod tetramethyl ester as a dark-purple solid.

To 4-carboxymethyl-rhod tetramethyl ester (128 mg, 0.15 mmol) in DMF (5 mL) and DIEA (0.40 mL, 2.2 mmol) was added dry TFA-SE (338 mg, 1.5 mmol). The mixture was stirred for 16 h, then a solution of 4-aminophenylethylamine (0.4 mL, 4 mmol) and DIEA (0.4 mL, 2.2. mmol) was introduced. The mixture was stirred for 2 h, diluted with $CHCl_3$ (500 mL), washed with 1% AcOH (3 x 100 mL), sat. NaCl (2 x 200 mL), filtered and evaporated. Ether (25 mL) was added to the residue, and the precipitated product was filtered and washed with ether to give 126 mg of 4-(4'-(aminophenyl)-2-ethylamino)carbonylmethyl-rhod tetramethyl ester as a dark-red solid.

To 4-(4'-(aminophenyl)-2-ethylamino)carbonylmethyl-rhod tetramethyl ester (100 mg, 0.1 mmol) in dioxane (2 mL), MeOH (2 mL) and H_2O (1 mL) was added 1 M KOH to give pH 12.0. The mixture was stirred for 50 h then the pH was adjusted to 9.0 with 0.1 M HCl. The mixture was evaporated and the residue was purified on Sephadex LH-20 using H_2O as eluant and the product lyophilized to give 21 mg of Compound 10 as an orange-red solid.

Example 34: Synthesis of BAPTA-agarose Compounds (Compounds 13 and 14)

Preparation of BAPTA-agarose (compound 13)

A solution of 5-isothiocyanato-BAPTA free acid (65 mg, 0.12 mmol, US Patent No. 5,453,517) in 3 mL anhydrous DMF was added to a slurry of amino agarose (50% aqueous slurry, 16 µmol amine/mL, 6 mL, 96 µmole amine, Pierce) that had been diluted with 15 mL DMF. The pH was raised to 10 with DIEA (1.5 mL). The resulting light-brown mixture was stirred at room temperature for 48 hours then centrifuged. The BAPTA-agarose (compound 13) pellet was rinsed with acetone (2x) and water (2x) then suspended in water.

Preparation of BAPTA-5F-agarose (Compound 14)

A solution of 5-amino-5'-fluoro-BAPTA free acid (0.26 g, 0.51 mmol) in 12 mL aqueous HCl was diluted with 12 mL chloroform then treated with thiophosgene (3 mL). The orange mixture was stirred at room temperature overnight then evaporated. The mixture was centrifuged, yielding a brown gum that was dried then dissolved in 2 mL anhydrous THF. Addition of 20 mL ethyl acetate gave a precipitate, which was isolated by centrifugation to give 5-fluoro-5'-isothiocyanoto-BAPTA free acid as a light gray-brown powder.

5-Fluoro-5'-isothiocyanato-BAPTA free acid (25 mg, 0.05 mmol) in 1 mL anhydrous DMF was added to 2 mL of a 50% aqueous slurry of amino agarose that had been diluted with 5 mL DMF. The pH was raised to 10 with a few drops of DIEA. The light-brown mixture was stirred at room temperature for 48 hours then centrifuged. BAPTA-5F-agarose (Compound 14) pellet was rinsed with acetone (2x) and water (2x) then suspended in water.

Example 35: Synthesis of Compound 15 (TAMRA-biotin BAPTA Compound)

A solution of BAPTA-4-isothiocyanate free acid (18 mg, 0.033 mmol) in 5 mL dioxane was added to a solution of 5-(and-6)-tetramethylrhodamine biocytin (Molecular Probes Inc., 29 mg, 0.033 mmol) in 4 mL water. The resulting pH (3.5) was raised to 10 with aqueous sodium carbonate. The resulting red solution was stirred at ambient temperature overnight, the concentrated *in vacuo*. The residue was purified by column chromatography on Sephadex LH-20, using water as eluant. The product was lyophilized to give TAMRA-biotin-BAPTA as 26 mg of red powder: LCMS m/2 726 (1452 calculated for $C_{73}H_{84}N_{11}O_{17}S_2$).

Example 36: Synthesis of Compound 16 (Rhodamine BAPTA compound)

5-Formyl-5'-nitro-BAPTA tetramethyl ester (200 mg, 0.33 mmol) and 8-hydroxyjulolidine (125 mg, 0.66 mmol) in 5 mL propionic acid was heated under nitrogen at 70 °C for 1 hour, cooled to room temperature and poured into 30 mL concentrated potassium acetate solution. The mixture was extracted with chloroform then washed with brine, dried over sodium sulfate, and evaporated to a red oil that was purified by flash chromatography using ethyl acetate/hexanes to give 0.225 g of dihydro-X-Rhod-5N tetramethyl ester as a yellow foam.

To dihydro-X-Rhod-5N tetramethyl ester (0.12 g, 0.12 mmol) in 1:1 chloroform/methanol (5 mL) was added chloranil (40 mg, 0.16 mmol). The solution was stirred overnight, diluted with

50 mL chloroform, washed with brine, dried over sodium sulfate, and evaporated. The residue was purified by flash chromatography using 15% methanol/chloroform to give 63 mg of X-Rhod-5N tetramethyl ester as a purple powder.

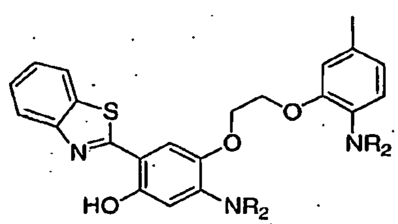
To X-Rhod-5N tetramethyl ester (0.11 g, 0.11 mmol) in 5 mL methanol was added 2 M KOH (0.6 mL, 1.2 mmol). The solution was stirred at room temperature overnight, then evaporated. The residue was dissolved in water (5 mL) and the pH lowered to 2 with 2 M HCl. A precipitate was collected by centrifugation, dissolved in fresh aqueous KOH and precipitated with aqueous HCl. This procedure was repeated five times to give 90 mg of Compound 16 free acid as a purple powder.

Example 37: Synthesis of 4-Hydroxy-5-benzothiazolyl-BAPTA (Compound 17)

A solution of 4-hydroxy-5-formyl-5'-methyl BAPTA, tetramethylester (0.40 g, 0.68 mmol) and 2-aminothiophenol (75 mg, 0.70 mmol) in DMSO (5 mL) was heated at reflux for 15 minutes. After cooling the yellow solution was diluted with 50 mL water. A yellow precipitate was filtered and dried, then purified by flash chromatography using ethyl acetate/hexanes to give 0.22 g of 4-hydroxy-5-benzothiazolyl-BAPTA tetramethylester as a yellow foam.

To 4-hydroxy-5-benzothiazolyl-BAPTA tetramethylester (0.21 g, 0.30 mmol) in 1:1 methanol/dioxane (10 mL) was added 1 M KOH (3.0 mL, 3.0 mmol). The solution was stirred for 3 hours then evaporated. The residue was purified on Sephadex LH-20 using water as eluant to give 0.13 g of compound 17 as a yellow-green powder ($R=CH_2CO_2K$).

Compound 17



Example 38: Synthesis of 4'-Carboxymethyl-4-methoxy-rhod, potassium salt (Compound 19)

A suspension of (4'-methoxy-2'-nitrophenoxy)-2-chloroethane (11.29 g, 48.7 mmol), methyl 4-hydroxy-3-nitrophenylacetate (10.80 g, 51.2 mmol), and K_2CO_3 (13.80 g, 100 mmol) was stirred at 130 °C for 4 h, cooled to room temperature, and poured into ice water (0.8 L), allowed to coagulate for 2 days. The precipitate was filtered, washed with H_2O and dried to give 15.1 g of (4'-methoxycarbonylmethyl-2'-nitrophenoxy)-2-(4"-methoxy-2"-nitrophenoxy)ethane as a yellow solid.

(4'-Methoxycarbonylmethyl-2'-nitrophenoxy)-2-(4"-methoxy-2"-nitrophenoxy)ethane (15.0 g, 43.3 mmol) was hydrogenated over 10% Pd/C (2.0 g) in CH_2Cl_2 (250 mL) at 45 psi for 16 h. The mixture was filtered through Celite. The filtrate was evaporated and the residue was treated with ether (200 mL). The precipitate was filtered and washed with ether (3 x 25 mL) to give 11.21 g of (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2"-amino-4"-methoxyphenoxy)ethane as off-white solid.

A mixture of (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2"-amino-4"-methoxyphenoxy)ethane (8.65 g, 25 mmol), methanol (80 mL), dioxane (80 mL), and 1 M KOH (50 mL, 50 mmol) was stirred at 60 °C for 1 h, then overnight at room temperature. The mixture was evaporated and the residue was suspended in H_2O (300 mL). Aqueous 1 M HCl was added to pH 4.0. The precipitate was filtered, washed with H_2O, and dried to give 6.84 g of (2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2"-amino-4"-methoxyphenoxy)ethane as off-white solid.

Diphenyldiazomethane was prepared by vigorously stirring benzophenone hydrazone (5.88 g, 30 mmol) and yellow HgO (17.60 g, 80 mmol) in hexanes (150 mL) for 6 h. The mixture was filtered from inorganics, filtrate was evaporated and the residue was re-dissolved in acetone (40 mL). This solution was added to the solution of (2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2"-amino-4"-methoxyphenoxy)ethane acid (6.64 g, 20 mmol) in acetone (200 mL). The resulting mixture was stirred for 48 h at 35 °C, evaporated and the residue was suspended in $CHCl_3$. To the suspension was added AcOH (4 mL) to decompose the excess reagent and the mixture was stirred for 2 h, then evaporated, and the crude product was purified by flash chromatography on SiO_2 using 0.5% MeOH in $CHCl_3$ as eluant to give (2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2"-amino-4"-methoxyphenoxy)ethane, 7.81 g (78%) as off-white solid. A mixture of diamine (2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2"-amino-4"-methoxyphenoxy)ethane (4.62 g, 9.3 mmol), DIEA (52 mL, 300 mmol), methyl bromoacetate (19 mL, 200 mmol), and NaI (0.75 g, 5 mmol) in MeCN (150 mL) was refluxed under stirring for 70 h, cooled to room

temperature and evaporated. The residue was dissolved in $CHCl_3$ (400 mL), washed with 1% AcOH (3 x 200 mL), H_2O (200 mL), sat. NaCl (2 x 200 mL), filtered and evaporated. The residue was purified by flash chromatography on SiO_2 using a gradient of 25–40% EtOAc in hexanes as eluant to give 3.01 g of 4-diphenylmethoxycarbonylmethyl-4'-methoxy-BAPTA tetramethyl ester as a colorless solid.

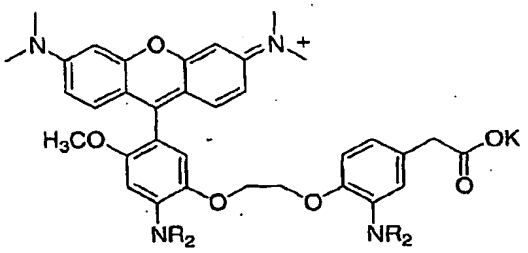
To a solution of Vilsmeier reagent made from $POCl_3$ (0.28 mL, 3 mmol) in DMF (2 mL) was added a solution of 4-diphenylmethoxycarbonylmethyl-4'-methoxy-BAPTA tetramethyl ester (762 mg, 1 mmol) in DMF (2 mL). The mixture was stirred for 2 h, then was quickly poured into an ice-sat. K_2CO_3 mixture (50 mL). The mixture was extracted with $CHCl_3$ (7 x 20 mL), dried over $MgSO_4$ and evaporated. The mixture of products was separated by column chromatography on SiO_2 (4 x 35 cm bed) using a gradient of 30–45% EtOAc in hexanes to give 760 mg of aldehyde 4-diphenylmethoxycarbonylmethyl-5'-formyl-4'-methoxy-BAPTA tetramethyl ester as a colorless solid.

A mixture of aldehyde 4-diphenylmethoxycarbonylmethyl-5'-formyl-4'-methoxy-BAPTA tetramethyl ester (1.58 g, 2.0 mmol), *m*-dimethylaminophenol (602 mg, 4.4 mmol), and TsOH (50 mg, catalyst) in propionic acid (20 mL) was stirred at 65 °C for 20 h, then cooled to room temperature and poured into 3 M NaOAc (300 mL). After 1 h, the precipitated product was filtered, washed with water, and dried to give 2.00 g of 4-diphenylmethoxycarbonylmethyl-5'dihydrorhod tetramethyl ester as a purple-red solid.

A mixture of compound 4-diphenylmethoxycarbonylmethyl-4'-methoxy-5'-dihydrorhod tetramethyl ester (2.00 g, 1.9 mmol) and powdered chloranil (0.50 g, 2 mmol) in $CHCl_3$ and MeOH (50 mL of each) was stirred for 4 h, filtered and evaporated. The residue was purified by flash chromatography on SiO_2 using a gradient of 5–7% MeOH in $CHCl_3$/ 0.5% AcOH to give a crude product, which was redissolved in $CHCl_3$, filtered from SiO_2, and evaporated to give 480 mg of 4-(diphenylmethoxycarbonylmethyl)-4'-methoxy-rhod, tetramethyl ester as a dark-purple solid.

To a solution of 4-(diphenylmethoxycarbonylmethyl)-4'-methoxy-rhod, tetramethyl ester (45 mg, 0.04 mmol) in dioxane (1 mL), MeOH (2 mL) and H_2O (2 mL) was added 1 M KOH to pH 12.0. The mixture was stirred for 50 h, then pH was adjusted to 9.0 with 0.1 M HCl. The mixture was evaporated and the residue was purified on Sephadex LH-20 column (2.6 x 90 cm bed) using H_2O as eluant and lyophilized to give 12 mg of Compound 19 as a red solid (R=CH_2CO_2K).

Compound 19



Example 39: Synthesis of Compound 20 Containing a DTPA Metal-chelating Moiety

BODIPY® TR cadaverine, Molecular Probes D-6251, 10 mg, 0.019 mmol was dissolved into a mixture of (*S*)-1-*p*-isothiocyanatobenzyldiethylenetriaminepentaacetic acid (DTPA isothiocyanate, Molecular Probes I-24221, 10 mg, 0.019 mmol) in 2 mL water. The pH was raised to 10 with aqueous sodium carbonate. The resulting blue solution was stirred at room temperature for two days, then concentrated *in vacuo*. The residue was purified by column chromatography on Sephadex LH-20 using E-pure water as eluant to give 2 mg of Compound 20 as a purple powder.

Example 40: Synthesis of Compound 21 containing a DTPA metal-chelating moiety

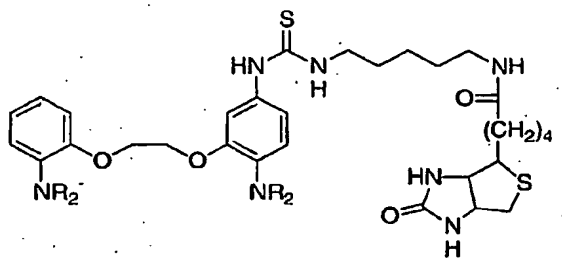
For the synthesis of carbamate **21a** a solution of penta-*t*-butyl 1-(*S*)-(*p*-aminobenzyl)-diethylenetriaminepentaacetate (prepared according to the published procedure of Donald T. Corson & Claude F. Meares. *Bioconjugate Chem.*, **11**(2), *2000*, 292-299; 0.800 g, 1.03 mmol) in 20 mL of methylene chloride was added 1 mL of pyridine followed by the addition of a solution of the acid chloride of *N*-CBZ-6-aminohexanoic acid (0.290 g, 1.02 mmol) in 5 mL of methylene chloride. The reaction mixture was stirred overnight at room temperature and concentrated in vacuo. The residue was dissolved in 100 mL of ethyl acetate and the resulting solution was washed with 10% HCl (2 x 30 mL), water (30 mL), brine (30 mL) and dried over sodium sulfate. The solution was concentrated and put on a silica gel column (packed with ethyl acetate). The column was eluted with ethyl acetate to remove impurities then the desired product was eluted with 10:1 chloroform-methanol. Pure fractions were combined and the solvent evaporated to give amide **21a** (0.54 g, 54%) as a viscous oil.

Example 41: Synthesis of Compound 22 Containing a DTPA Metal-chelating Moiety

BODIPY® FL EDA, Molecular Probes D-2390, 7 mg, 0.019 mmol was dissolved into a mixture of DTPA isothiocyanate, Molecular Probes I-24221, 10 mg, 0.019 mmol in 2 mL water. The pH was raised to 10 with aqueous sodium carbonate. The resulting orange solution is stirred at room temperature for 3.5 hours, then evaporated. The residue was purified by on Sephadex LH-20 using water as eluant to give29 mg of Compound 22 as an orange powder.

Example 42. Synthesis of Compound 25 – A BAPTA-Biotin

To a solution of biotin-cadaverine (21 mg, 0.047 mmol, Molecular Probes) in 2 mL water was added 2 drops saturated sodium carbonate solution. A solution of 5-isothiocyanato-BAPTA free acid (25 mg, 0.047 mmol) in 3 mL dioxane was added. The reaction pH was raised to 9.5 with more sodium carbonate solution, and the solution was stirred overnight at ambient temperature. The volatiles were removed in vacuo and the residue was purified by chromatographpy on Sephadex LH-20 using water as eluant to give compound 25 as 40 mg of a pale brown powder (R=CH_2CO_2Na).

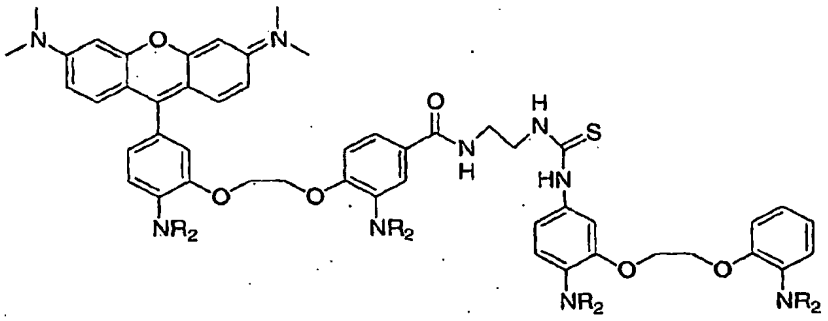


Compound 25

Example 43. Synthesis of Compound 26 – A Rhod-BAPTA-BAPTA

To a solution of the ethylenediamine carboxamide of 4-carboxy-rhod tetramethyl ester (as in example 34, 0.04 mmol) in 1:1 water:dioxane (5 mL) was added a solution of 5-isothiocyanato-BAPTA free acid (24 mg, 0.044 mmol) in 1:1 water:dioxane (8 mL). The pH was raised to 8.5 by addition of aqueous sodium bicarbonate. The resulting red solution was

stirred at ambient temperature overnight, then concentrated in vacuo and purified by column chromatography on Sephadex LH-20 using water as eluant to give the intermediate tetramethyl ester tetracarboxylate as 11 mg of red powder. To a solution of this intermediate (0.007 mmol) in 1.4 mL water was added 1M KOH (0.07 mmol). After 3 hours the pH (13) was lowered to 9 with aqueous acetic acid, followed by concentration in vacuo. The resulting residue was purifed by column chromatography on Sephadex LH-20 using water as eluant to afford compound 26 as 8 mg of a red powder (R=CH_2CO_2K).



Compound 26

The reagents employed in the preceding examples are commercially available or can be prepared using commercially available instrumentation, methods, or reagents known in the art or whose preparation is described in the examples. It is evident from the above description and results that the subject invention is greatly superior to presently available methods for labeling phosphorylated target molecules in a biological sample, as an unprecedented 500–1000 fold concentration range of phosphorylated target molecules can be detected. The subject invention overcomes the shortcomings of the currently used methods by allowing labeling as well as isolation of phosphorylated target molecules in a simple procedure that has increased sensitivity. It is appreciated that the methods of the present invention provide labeling of phosphorylated target molecules in solution or immobilized and that the phosphate-binding compounds can be either immobilized or in solution, allowing for identification of enzymes responsible for phosphorylation of these target molecules. The examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the foregoing invention has been described in extensive detail by way of illustration and example for purposes of clarity for understanding, those of ordinary skill in the art will readily realize that many changes and

modifications can be made thereto without departing from the spirit or scope of the appended claims.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

What is claimed is:

1. A binding solution comprising:

   a) a phosphate-binding compound having formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4;

   b) a salt comprising metal ions; and,

   c) an acid.

2. The binding solution according to Claim 1, wherein said binding solution optionally further comprises an organic solvent and a buffering agent.

3. The binding solution according to Claim 2, wherein said organic solvent is acetonitrile.

4. The binding solution according to Claim 1, wherein said phosphate-binding compounds are immobilized on a solid or semi-solid matrix.

5. The binding solution according to Claim 3, wherein said binding solution has a pH about 3 to about pH 6.

6. The binding solution according to Claim 5, wherein said metal ion is selected from the group consisting of Ga^{3+}, Fe^{3+} and Al^{3+}.

7. The binding solution according to Claim 6, wherein said salt is gallium chloride.

8. The binding solution according to Claim 5, wherein said chemical moiety is a label that is a dye, an enzyme or a hapten provided that the dye is not sulfonated.

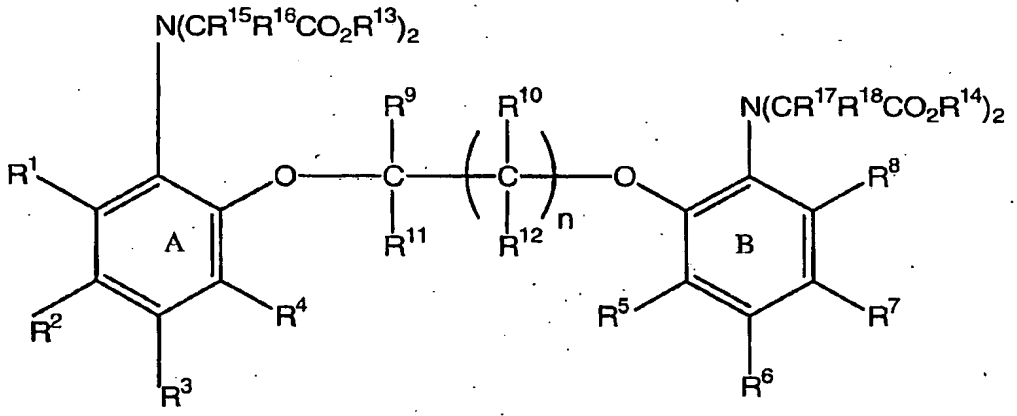
9. The binding solution according to Claim 5, wherein said chemical moiety is a reactive group.

10. The binding solution according to Claim 9, wherein said hapten is biotin.

11. The binding solution according to Claim 9, wherein said dye is selected from the group consisting of a benzofuran, a quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

12. The binding solution according to Claim 9, wherein said metal-chelating moiety is BAPTA.

13. The binding solution according to Claim 12, wherein said compound has the formula (A)m(L)n(B) wherein A is a chemical moiety that is a dye or a reactive group, L is a linker, m is an integer from 1 to 4, n is an integer from 0 to 4 and B is a metal-chelating moiety having said Formula IV comprising;



FORMULA IV

wherein said dye or reactive group is attached to at least one of R^1-R^{12} by a linker or at least one of R^1-R^8 in combination with ring A or ring B forms a dye;

R^1-R^8 that are not a dye or reactive group are independently selected from the group consisting of hydrogen, halogen, alkoxy, alkyl, aryl, amino, carboxyl, nitro, cyano, thioether, hydroxyl, sulfinyl and linker;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen, linker and lower alkyl, or adjacent substituents R^9 and R^{10} in combination constitute a 5-membered or 6-membered alicyclic or heterocyclic ring;

R^{15}, R^{16}, R^{17} and R^{18} are independently hydrogen, lower alkyl or alkyl, wherein alkyl or lower alkyl is optionally substituted by carboxyl or alkoxy;

p is 1 or 2; and,

R^{13} and R^{14} are independently hydrogen or a salt.

14. The binding solution according to Claim 13, wherein at least one of said R^1-R^4 is independently a dye or reactive group and R^5-R^8 is independently selected from the group consisting of H, NO_2, F, CF_3, lower alkyl, and linker wherein said linker is optionally attached to a biotin, a reactive group or a dye.

15. The binding solution according to Claim 14, wherein said dye is independently R^3, R^2 or R^3 and R^2 together.

16. The binding solution according to Claim 13, wherein said R^6 or R^7 is a linker and said linker is optionally attached to a biotin, a reactive group or dye.

17. The binding solution according to Claim 13, wherein said R^6 and R^5 are independently fluorine.

18. The binding solution according to Claim 13, wherein said R^6 is NO_2.

19. The binding solution according to Claim 13, wherein said phosphate-binding compounds having said formula (A)m(L)n(B) are selected from the group consisting of Compound 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12.

20. The binding solution according to Claim 19, wherein said compounds are selected from the group consisting of Compound 1, 2, 3, 4, 5, 8, 9, 10, 11 and 12.

21. The binding solution according to Claim 20, wherein said compounds are selected from the group consisting of Compound 1, 2, 5 and 10.

22. A method for binding a phosphorylated target molecule in a sample, said method comprising the steps of:

i) contacting said sample with a binding solution, wherein said binding solution comprises,

a) a phosphate-binding compound having formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4;

b) a salt comprising metal ions;

c) an acid; and,

ii) incubating said sample and said binding solution for sufficient time to allow said phosphate-binding compound to associate with said phosphorylated target molecule whereby said phosphorylated target molecule is bound.

23. The method according to Claim 22, wherein said method further comprises illuminating said phosphate-binding compound with a suitable light source whereby said labeled phosphorylated target molecule is detected.

24. The method according to Claim 22, wherein said phosphorylated target molecule is selected from the group consisting of proteins, peptides, nucleotides, carbohydrates, phosphatase substrates, kinase substrates, lipids and inorganic phosphate.

25. The method according to Claim 24, wherein said phosphorylated target molecules are immobilized on a solid or semi-solid matrix or are solubilized in solution.

26. The method according to Claim 22, wherein said phosphate-binding compound is immobilized on a solid or semi-solid matrix.

27. The method according to Claim 25 or 26, wherein said solid or semi-solid matrix is a gel, a membrane, a polymeric particle, or an array.

28. The method according to Claim 27, wherein said binding solution has a pH about 3 to about pH 6.

29. The method according to Claim 28, wherein said metal ion is selected from the group consisting of Ga^{3+}, Fe^{3+} and Al^{3+}.

30. The method according to Claim 29, wherein said salt is gallium chloride.

31. The method according to Claim 30, wherein said metal-chelating moiety is BAPTA.

32. The method according to Claim 31, wherein said chemical moiety is a reactive group or a label that is a dye, an enzyme or a hapten provided that the dye is not sulfonated.

33. The method according to Claim 32, wherein said hapten is a biotin.

34. The method according to Claim 32, wherein said dye is selected from the group consisting of a benzofuran, a quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

35. A method for detecting a phosphorylated target molecule in a sample immobilized on a membrane, said method comprising the steps of:

i) optionally electrophoretically separating said sample on a gel;
ii) immoblilizing said sample to a membrane;
iii) optionally contacting said membrane of step ii) with a fixing solution;
iv) contacting said membrane of step iii) with a binding solution, wherein said binding solution comprises;
    a) a phosphate-binding compound having formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4;
    b) a salt comprising metal ions; and,
    c) an acid;
v) incubating said membrane of step iv) and said binding solution for sufficient time to allow said phosphate-binding compound to associate with said phosphorylated target molecule; and,
vi) visualizing said compound whereby said phosphorylated target molecule is detected.

36. The method according to Claim 35, wherein step iii) further comprises contacting said membrane with a wash solution following contact with said fixing solution.

37. The method according to Claim 35, wherein said phosphorylated target molecule is selected from the group consisting of proteins, peptides, and nucleotides.

38. The method according to Claim 35, wherein said binding solution has a pH about 3 to about pH 6.

39. The method according to Claim 38, wherein said metal ions are selected from the group consisting of Ga^{3+}, Fe^{3+} and Al^{3+}.

40. The method according to Claim 39, wherein said salt is gallium chloride.

41. The method according to Claim 40, wherein said metal-chelating moiety comprises Formula VI.

42. The method according to Claim 41, wherein said chemical moiety is a label that is a dye, an enzyme or a hapten.

43. The method according to Claim 42, wherein said dye is selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

44. The method according to Claim 41, wherein said phosphate-binding compounds having said formula (A)m(L)n(B) wherein B comprises Formula VI are selected from the group consisting of Compound 1, 2, 3, 4, 5, 9, 10, 11 and 12.

45. The method according to Claim 45, wherein said compound is Compound 2.

46. The method according to Claim 41, wherein said step v) further comprises contacting said membrane with a wash solution following contact with said binding solution.

47. The method according to Claim 46, further comprising adding an additional detection reagent to said membrane.

48. The method according to Claim 49, wherein said additional detection reagent is a staining solution specific for total proteins or a staining solution specific for glycoproteins.

49. A method for detecting a phosphorylated target molecule in a sample immobilized on a gel, said method comprising the steps of:

i) immobilizing said sample on a gel;
ii) optionally contacting said gel of step i) with a fixing solution;
iii) contacting said gel of step ii) with a binding solution, wherein said binding solution comprises;
a) a compound having formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4;
b) a salt comprising metal ions; and,
c) an acid ;
iv) incubating said gel of step iii) and said binding solution for sufficient time to allow said compound to associate with said phosphorylated target molecule; and,
v) visualizing said compound whereby said phosphorylated target molecule is detected.

50. The method according to Claim 49, wherein step ii) further comprises contacting said gel with a wash solution following contact with said fixing solution.

51. The method according to Claim 49, wherein said method further comprises first electrofocusing said sample before step i.

52. The method according to Claim 49, wherein said step i of said method further comprise electrophoretically separating said sample.

53. The method according to Claim 49, wherein said phosphorylated target molecules are selected from the group consisting of proteins, peptides, and nucleotides.

54. The method according to Claim 53, wherein said binding solution has a pH about 3 to about pH 6.

55. The method according to Claim 54, wherein said metal ions are selected from the group consisting of Ga^{3+}, Fe^{3+} and Al^{3+}.

56. The method according to Claim 55, wherein said salt is gallium chloride.

57. The method according to Claim 56, wherein said metal-chelating moiety comprises Formula VI.

58. The method according to Claim 57, wherein said chemical moiety is a reactive group or a label that is independently a dye, an enzyme or a hapten.

59. The method according to Claim 58, wherein said dye is selected from the group consisting of a benzofuran, a quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

60. The method according to Claim 58, wherein said step v) further comprises contacting said gel with a wash solution following contact with said binding solution.

61. The method according to Claim 59, wherein said phosphate-binding compounds having said formula (A)m(L)n(B) wherein B comprises Formula VI are selected from the group consisting of Compound 1, 2, 3, 4, 5, 9, 10, 11 and 12.

62. The method according to Claim 62, wherein said compound is Compound 2.

63. The method according to Claim 60, further comprising adding an additional detection reagent to said membrane.

64. The method according to Claim 63, wherein said additional detection reagent is a staining solution specific for total proteins or a staining solution specific for glycoproteins.

65. A method for detecting a phosphorylated target molecule in a sample immobilized on a matrix, said method comprising the steps of:

i) immobilizing said sample on an matrix;

ii) contacting said matrix of step i) with a binding solution, wherein said binding solution comprises;

a) a phosphate-binding compound having formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal-chelating moiety, m is an integer from 1 to 4 and n is an integer from 0 to 4;

b) a salt comprising metal ions; and,

c) an acid;

iii) incubating said matrix of step ii) and said binding solution for sufficient time to allow said compound to associate with said phosphorylated target molecule; and,

iv) visualizing said compound whereby said phosphorylated target molecule is detected.

66. The method according to Claim 65, wherein said sample is selected from the group consisting of proteins, peptides, nucleotides, lipids, kinase substrates, phosphatase substrates, phosphate binding proteins and carbohydrates.

67. The method according to Claim 66, wherein said matrix is a polymeric gel, glass, polymeric membrane, plastic, polymeric microparticle.

68. The method according to Claim 66, wherein said binding solution has a pH about 3 to about pH 6.

69. The method according to Claim 68, wherein said metal ion is selected from the group consisting of Ga^{3+}, Fe^{3+} and Al^{3+}.

70. The method according to Claim 69, wherein said salt is gallium chloride.

71. The method according to Claim 70, wherein said metal-chelating moiety comprises Formula VI.

72. The method according to Claim 71, wherein said chemical moiety is a reactive group or a label that is a dye, an enzyme or a hapten provided that the dye is not sulfonated.

90. The method according to Claim 89, wherein said dye is selected from the group consisting of a benzofuran, a quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

91. The method according to Claim 89, wherein said hapten is biotin.

92. The method according to Claim 89, wherein said compound having said formula (A)m(L)n(B) wherein B is Formula IV are selected from the group consisting of compound 1, 2, 3, 4, 5, 6, 9, 10, 12, 15, and 16.

93. A method for isolating phosphorylated target molecules from a sample, said method comprising the steps of:

i) charging a matrix comprising a metal-chelating moiety selected from the group consisting of Formula IV with a salt comprising metal ions;
ii) equilibrating said matrix with an acidic binding buffer,
iii) adding said sample to said matrix wherein said phosphorylated target molecules are bound to said matrix of step ii);
iv) eluting said phosphorylated target molecules from said matrix with a base solution whereby said phosphorylated target molecules are isolated.

94. The method according to Claim 93, wherein said matrix is selected from the group consisting of polymeric particles, polymeric membrane, glass and plastic.

95. The method according to Claim 94, wherein said phosphorylated target molecules are proteins, peptide or nucleotides.

96. The method according to Claim 95, wherein said acidic binding buffer has a pH about 3 to about 6.

97. The method according to Claim 96, wherein said metal ions are selected from the group consisting of Ga^{3+}, Fe^{3+} and Al^{3+}.

98. The method according to Claim 97, wherein said salt is gallium chloride.

hydroxyl, sulfinyl and linker;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen, linker and lower alkyl, or adjacent substituents R^9 and R^{10} in combination constitute a 5-membered or 6-membered alicyclic or heterocyclic ring;

R^{15}, R^{16}, R^{17} and R^{18} are independently hydrogen, lower alkyl or alkyl, wherein alkyl or lower alkyl is optionally substituted by carboxyl or alkoxy;

n is 1 or 2; and,

R^{13} and R^{14} are independently hydrogen or a salt.

112. The kit according to Claim 111, wherein at least one of said R^1-R^4 is independently a dye label or a reactive group and R^5-R^8 is independently selected from the group consisting of H, NO_2, F, CF_3, lower alkyl, and linker wherein said linker is optionally attached to a biotin, reactive group or dye label.

113. The kit according to Claim 112, wherein said dye label is independently R^3, R^2 or R^3 and R^2 together.

114. The kit according to Claim 112, wherein said R^6 or R^7 is a linker and said linker is optionally attached to a biotin, a reactive group or dye label.

115. The kit according to Claim 112, wherein said R^6 and R^5 are independently fluorine.

116. The kit according to Claim 112, wherein said R^6 is NO_2.

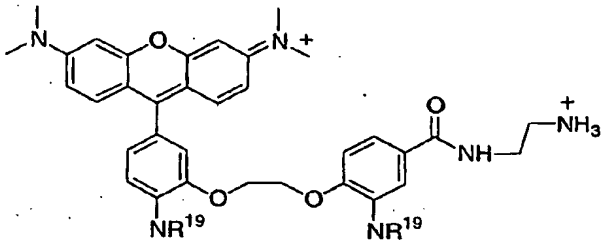
117. The kit according to Claim 111, wherein said phosphate-binding compounds having said formula (A)m(L)n(B) are selected from the group consisting of Compound 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15 and 16.

118. The kit according to Claim 117, wherein said compounds are selected from the group consisting of Compound 1, 2, 5 and 10.

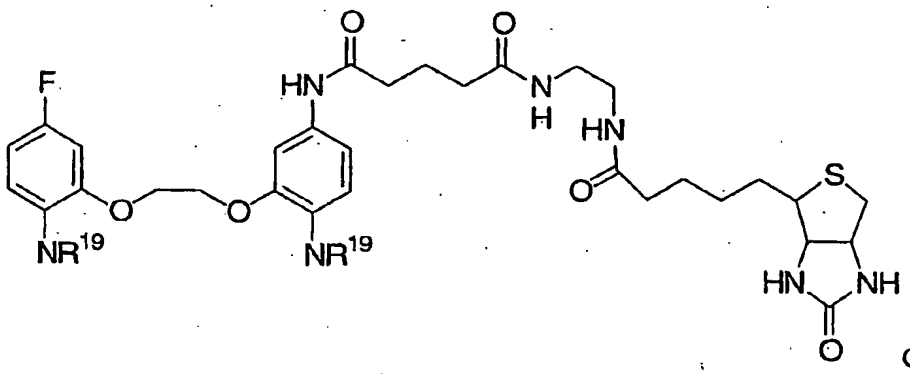
119. A matrix comprising an immobilized phosphate-binding compound according to Formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal

$\overset{|}{N}R^{19}$ $\overset{|}{N}R^{19}$

Compound 10;



Compound 11;



Compound 12;

124. A ternary complex comprising a trivalent gallium ion, a phosphorylated target molecule and a phosphate-binding compound according to formula (A)m(L)n(B) wherein A is a chemical moiety, L is a linker, B is a metal chelating moiety, m is an interger from 1 to 4 and n is an interger from 0 to 4.

Figure 2A

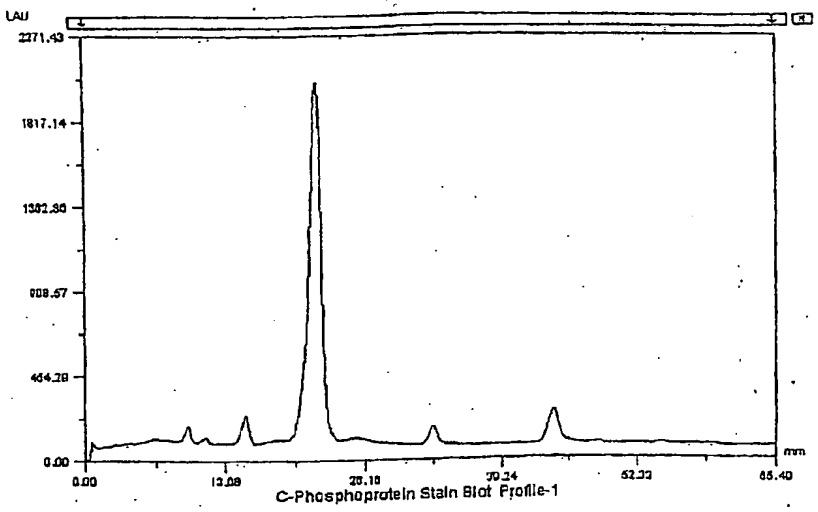


Figure 2B

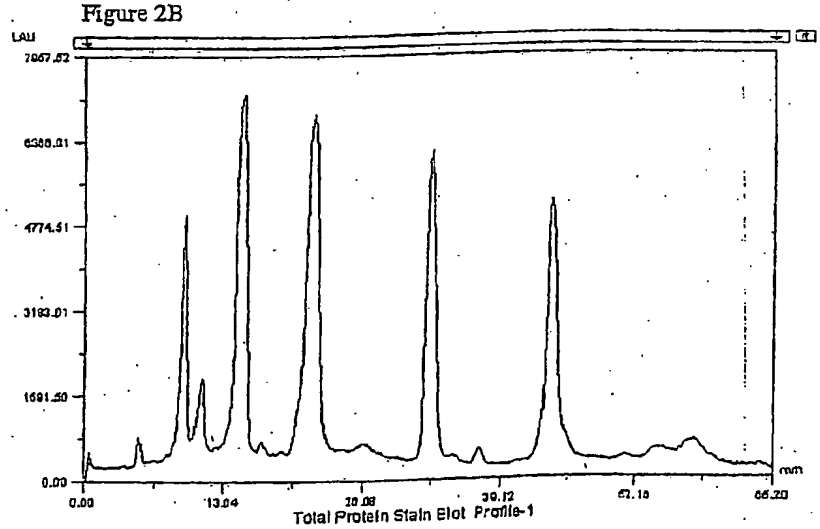


Figure 5

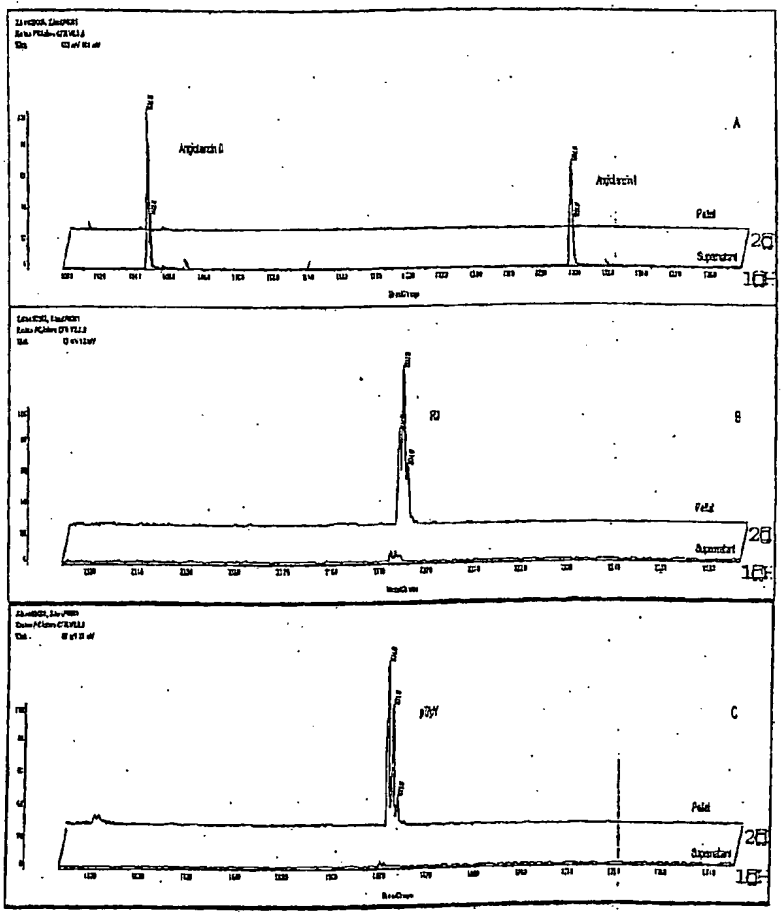


Figure 10A)

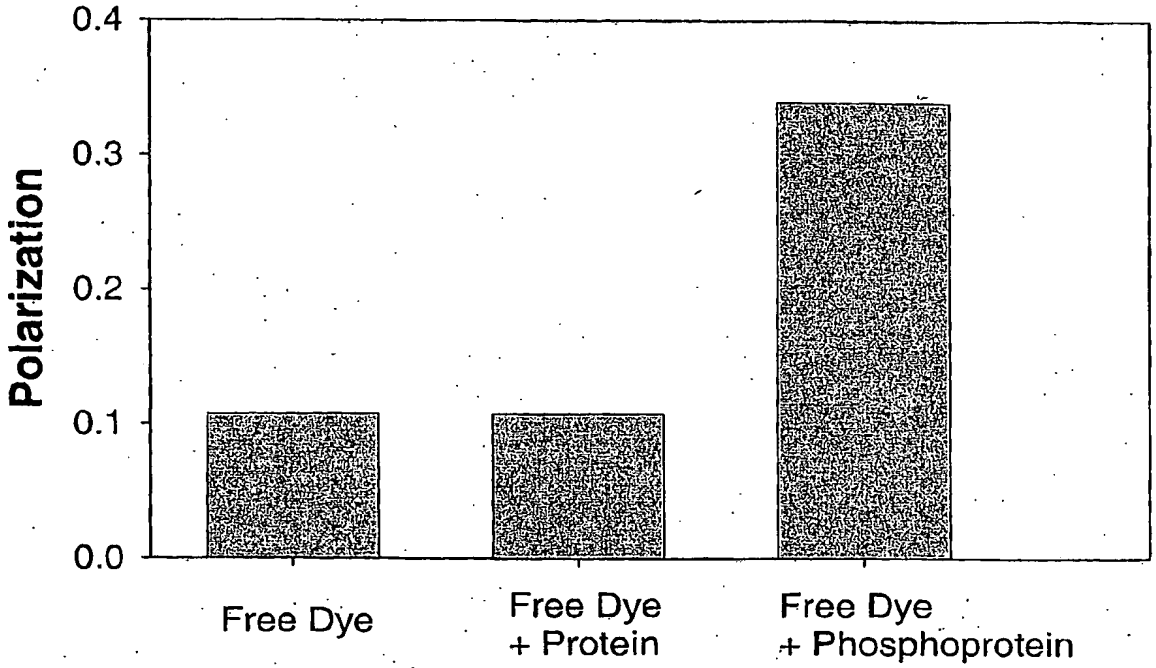


Figure 10B)